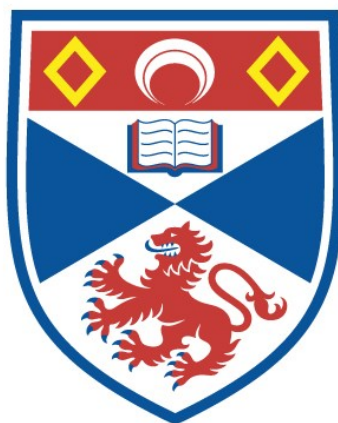


MOLECULAR CHARACTERISATION OF UREAPLASMA UREALYTICUM

Alison D. Myles

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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MOLECULAR CHARACTERISATION OF UREAPLASMA UREALYTICUM

A thesis presented by Alison D. Myles to the Univerity of St Andrews
in application for the Degree of Doctor of Philosophy.

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DECLARATION.

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry and Microbiology, University of St Andrews under the direction of Dr. D. Thirkell.

ACADEMIC RECORD.

I first matriculated at the Glasgow College of Technology in October 1981 and graduated with the Degree of Bachelor of Science, upper second class honours in Applied Biology (Microbiology) in November 1986.

I matriculated as a research student in the Department of Biochemistry and Microbiology, University of St Andrews in October 1986.

CERTIFICATE.

I hereby certify that Alison Denise Myles has spent nine terms engaged in research work under my direction and she has fulfilled the conditions of Ordinance General No 12, and Resolution of The University Court 1967, No 1 and that she has qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

ACKNOWLEDGEMENT.

I would like to thank Dr. D. Thirkell, Professor W.C. Russell and Mr. B. Precious for all their advice and encouragement throughout the 3 years. I would also like to thank Katie and John for putting up with me in the lab, especially during disasters.

Thanks also go to Mr. I. Armitt and Mr. I. Davidson for their technical assistance. I am also indebted to Mr. B. Blyth for producing the photographs for this thesis.

I would also like to acknowledge the financial support which was provided by the B.T.G.

Special thanks go to James for his continuing support over the last 3 years and especially for his help with the computing of this thesis.

DEDICATION.

I would like to dedicate this thesis to my parents, but especially to my father who insisted that I study science.

ABSTRACT.

Monoclonal antibodies (Mabs) raised against *Ureaplasma urealyticum* (serotype 8) revealed the presence of three membrane antigens. One major surface antigen of apparent molecular mass 96 kDa, shown to express four distinct epitopes, was found to be serotype-8 specific. Thus, Mabs raised against this polypeptide will unequivocally differentiate serotype 8 from the other serotypes of human origin. The binding of antibodies to this polypeptide partially suppressed the growth of the organisms. Membrane expressed antigenic polypeptides of apparent molecular masses 16 kDa and 17 kDa were expressed by those serotypes belonging to the large serocluster (A), whereas the 17 kDa polypeptide only was expressed in the smaller serocluster (B). Using this Mab probe serotype 13 was placed in the larger serocluster. Thus, Mabs which recognise one or both of these polypeptides will unequivocally differentiate the two seroclusters of this organism. The cytosolic urease from *U. urealyticum*, serotype 8, was purified by immuno-affinity chromatography. Two active forms of the enzyme were demonstrated by non-denaturing electrophoretic analysis and a single peak with urease activity of apparent molecular mass 190 kDa was shown by FPLC. Freezing and thawing of the purified enzyme caused a partial breakdown to inactive sub-units whereas total inactivation of the enzyme and denaturation, achieved by boiling for two minutes in the absence of any added denaturing agents, revealed three subunit polypeptides of apparent molecular masses 72, 14 and 11 kDa. Densitometry suggested that the active enzyme contains equimolar ratios of the three subunits and hence is a hexamer. The active enzyme displayed two pH optima of 6.9 and 6.15. Mabs raised against purified urease bound to both the active enzyme and to the inactive 72 kDa subunit. No evidence of antigenicity was found for the 14 and 11 kDa sub-units. These Mabs cross-reacted with ureases from all the other human serotypes. Competition assays revealed a minimum of four and possibly five distinct epitopes on the enzyme, all distinct from its active site.

Ureaplasmas from 5 animal hosts were studied using the various Mabs. The 96 kDa antigen was not found in any of the non-human strains. Variations in the available epitopes on the ureases and the presence or absence of the 16/17 kDa antigens in the non-human strains allowed a putative identification of the source of the non-human ureaplasmas. Such investigations also showed that with the exception of the 96 kDa serotype 8-specific antigen, chimpanzee isolates could not be differentiated from the human ureaplasma serotypes belonging to the large serocluster.

These Mabs were also used to develop fluorescent probes and other diagnostic assays which included a slide agglutination system and a sensitive urease catch assay which was also converted into a 'dip-stick' assay.

CONTENTS.

1	<u>INTRODUCTION.</u>	1
1.1	TAXONOMY	2
1.1.1	SEROLOGY OF <i>UREAPLASMA UREALYTICUM</i> .	7
1.1.1.1	Metabolic Inhibition (M.I.) Test.	7
1.1.1.2	Growth Inhibition (G.I.).	9
1.1.1.3	Immunofluorescence.	10
1.1.1.4	Indirect Immunoperoxidase.	11
1.1.1.5	DNA Homology.	12
1.1.1.6	Enzyme Linked Immunosorbent Assay (ELISA).	13
1.1.1.7	Isoelectric Focussing and Polyacrylamide gel Electrophoresis (PAGE).	13
1.1.1.8	Immunobinding.	15
1.1.1.9	Indirect Haemagglutination Test.	15
1.1.1.10	Direct Test For Urease.	15
1.2	CELL MORPHOLOGY AND SIZE.	16
1.2.1	THE CELL MEMBRANE OF UREAPLASMAS.	18
1.3	REPRODUCTION.	21
1.4	CELL GROWTH	22
1.5	METABOLISM	26
1.6	PATHOGENICITY	30
1.6.1	NON GONOCOCCAL URETHRITIS (N.G.U.)	31
1.6.2	INFERTILITY.	35
1.6.3	ARTHRITIS.	38
1.6.4	PREGNANCY DISORDERS.	39
1.6.4.1	CHORIOAMNIONITIS.	39
1.6.4.2	SPONTANEOUS ABORTION AND STILLBIRTH.	41

1.6.4.3	LOW BIRTH WEIGHT.	44
1.6.4.4	PELVIC INFLAMMATORY DISEASE (PID).	49
1.6.4.5	POSTPARTUM FEVER.	50
1.6.5	UPPER URINARY TRACT DISEASE.	51
1.6.5.1	URINARY TRACT STONES.	51
1.6.6	REFLUX NEPHROPATHY (CHRONIC ATROPIC PYELONEPHRITIS).	54
1.7	VIRULENCE MECHANISMS.	55
1.7.1	IMMUNOGLOBULIN A PROTEASE.	55
1.7.2	UREASE.	57
1.7.3	PHOSPHOLIPASES.	57
1.7.4	CELLULAR COMPOSITION.	58
1.7.5	ANTIBIOTIC RESISTANCE.	59
1.8	MOLECULAR BIOLOGY AND GENETICS OF MYCOPLASMAS (REVIEWED BY RAZIN, 1985).	60
1.8.1	GENOME REPLICATION	62
1.8.2	TRANSFORMATION AND TRANSFECTION.	63
1.8.3	TRANSCRIPTION.	64
1.8.3.1	RNA POLYMERASES.	64
1.8.3.2	RIBOSOMES	64
1.8.3.3	TRANSFER RNA (tRNA).	65
1.8.4	CODON USAGE.	66
1.8.5	PLASMIDS AND VIRUSES.	66
1.9	AIMS OF THIS RESEARCH.	68
	<u>2 MATERIALS AND METHODS.</u>	69
2.1	ORGANISMS USED.	70
2.2	MEDIUM AND CELL PRODUCTION.	71
2.3	RADIOIMMUNE ASSAY (RIA).	72
2.4	ANTIBODY PRODUCTION.	72

2.5	PROTEIN ESTIMATIONS.	77
2.6	POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE).	78
2.6.1	SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).	78
2.6.2	NON-DENATURED PAGE.	79
2.7	IMMUNOBLOTTING.	79
2.8	COMPETITION ASSAYS.	80
2.8.1	PURIFICATION OF MONOCLONAL ANTIBODIES.	80
2.8.2	RADIOLABELLING OF THE MONOCLONAL ANTIBODIES.	81
2.8.3	TITRATION OF LABELLED MONOCLONAL ANTIBODIES.	81
2.8.4	ASSAY PROCEDURES.	82
2.9	INHIBITION OF METABOLISM WITH ANTI-96 kDa AND WITH ANTI-16/17 kDa MABS.	83
2.10	PURIFICATION OF 96, 17, 16 kDa MEMBRANE ANTIGENS FROM <i>UREAPLASMA UREALYTICUM</i> , SEROTYPE 8.	84
2.10.1	PREPARATION OF AFFINITY COLUMNS (Axen <i>et al.</i> , 1967).	84
2.10.2	PURIFICATION OF THE 96 kDa MEMBRANE ANTIGEN.	84
2.10.3	PURIFICATION OF THE 16, 17 kDa ANTIGENS.	85
2.11	PURIFICATION OF UREASE FROM SEROTYPE 8.	85
2.11.1	PREPARATION OF AFFINITY COLUMN.	85
2.11.2	PURIFICATION (Following methodology previously described by Precious <i>et al.</i> , 1987).	86
2.12	DETECTION OF GLYCOPROTEINS (Knapp, P., 1978).	86
2.13	PHASE PARTITIONING OF THE INTEGRAL MEMBRANE AND CYTOSOLIC PROTEINS OF <i>UREAPLASMA UREALYTICUM</i> , SEROTYPES 1, 7 AND 8 USING TRITON X-114, (TX-114).	87
2.14	LABELLING OF <i>UREAPLASMA UREALYTICUM</i> , SEROTYPE 8, WITH ¹²⁵ I-LABELLED BOLTON AND HUNTER REAGENT (N-succinimidyl 3-(4-hydroxy-5-iodophenyl)propionate).	88
2.15	IMMUNOPRECIPITATIONS.	88
2.16	LABELLING OF <i>UREAPLASMA UREALYTICUM</i> , SEROTYPE 8, WITH (9,10(n)- ³ H) PALMITIC ACID.	89
2.17	DETECTION OF UREASE ACTIVITY ON GELS AFTER SDS-PAGE OR AFTER NON-DENATURING PAGE.	90

2.18 DETERMINATION OF THE MOLECULAR MASS OF IMMUNOAFFINITY PURIFIED UREASE OF SEROTYPE 8 BY GEL FILTRATION (FPLC).	91
2.19 UREASE SUB-UNIT RATIO BY DENSITOMETRY.	91
2.20 pH ACTIVITY OF THE UREASE FROM <i>UREAPLASMA UREALYTICUM</i> , SEROTYPE 8.	92
2.21 UREASE CATCH TEST.	92
2.21.1 SENSITIVITY OF THE CATCH TEST.	93
2.22 ELECTRON MICROSCOPY, THE ULTRASTRUCTURE OF <i>U. UREALYTICUM</i>	93
2.23 IMMUNOCYTOCHEMISTRY.	94
2.24 IMMUNOFLUORESCENCE.	96
2.24.1 INDIRECT METHOD.	96
2.24.2 DIRECT METHOD.	97
2.24.3 SENSITIVITY OF THE FITC-CONJUGATED MAB'S.	99
2.25 CLINICAL SAMPLES.	100
2.25.1 CONTROL INVESTIGATIONS.	102
2.26 THE DETECTION OF UREASE FROM UREAPLASMAS USING A 'DIP STICK'	102
2.26.1 SENSITIVITY OF THE 'DIP STICK'.	103
2.27 SLIDE AGGLUTINATION.	103
 <u>3 THREE MEMBRANE ANTIGENS OF <i>UREAPLASMA UREALYTICUM</i>, SEROTYPE 8.</u>	 105
3.1 THE SEROTYPES OF <i>U. UREALYTICUM</i> .	106
3.1.1 ELECTROPHORETIC ANALYSIS OF THE SEROTYPES.	108
3.2 THE 96 kDa ANTIGEN.	109
3.2.1 COMPETITION ASSAYS.	109
3.2.2 INHIBITION OF METABOLISM BY ANTI-96 kDa MABS.	112
3.2.3 PURIFICATION OF THE 96kDa ANTIGEN OF SEROTYPE 8.	123
3.2.4 SEROTYPE 8 SPECIFICITY OF THE 96 kDa ANTIGEN.	124
3.3 THE 16 kDa AND 17 kDa ANTIGENS OF <i>UREAPLASMA UREALYTICUM</i>	124
3.3.1 INHIBITION OF METABOLISM.	124

3.3.2	PURIFICATION OF THE 16 kDa AND 17 kDa POLYPEPTIDES.	131
3.3.3	THE 16 kDa AND 17 kDa POLYPEPTIDES HAVE ANTIGENS WHICH ARE SEROCLUSTER-SPECIFIC.	134
3.4	96/16/17/ kDa POLYPEPTIDES ARE MEMBRANE ANTIGENS.	134
3.5	UPTAKE OF ³ H-PALMITIC ACID BY <i>U. UREALYTICUM</i> .	144
3.5.1	IDENTIFICATION OF INTEGRAL MEMBRANE PROTEINS OF <i>U. UREALYTICUM</i> BY MABS AND HOMOLOGOUS POLYCLONAL ANTISERUM.	150
<u>4</u>	<u>THE UREASE ENZYME OF <i>UREAPLASMA UREALYTICUM</i>.</u>	151
4.1	THE UREASE ENZYME.	152
4.2	SUBUNIT STRUCTURE OF UREAPLASMA UREASE.	152
4.3	STABILITY OF THE ENZYME.	156
4.4	pH OPTIMA FOR ENZYME ACTIVITY.	156
4.5	THE UREASES OF <i>U. UREALYTICUM</i> , SEROTYPES 1-14.	159
4.6	UREASE CATCH ASSAY.	162
4.7	ANTI-UREASE MABS.	164
4.8	CROSS-REACTIONS OF MABS WITH THE UREASE OF THE OTHER 13 SEROTYPES OF <i>U. UREALYTICUM</i> .	165
4.9	COMPETITION ASSAYS FOR DETERMINATION OF EPITOPES ON THE UREASE.	170
4.10	CELLULAR LOCATION OF UREAPLASMA UREASE.	177
<u>5</u>	<u>THE ULTRASTRUCTURE OF <i>UREAPLASMA UREALYTICUM</i>, SEROTYPE 8.</u>	181
5.1	THE ULTRASTRUCTURE OF <i>U. UREALYTICUM</i> , SEROTYPE 8.	182
5.2	IMMUNOCYTOCHEMISTRY.	188
<u>6</u>	<u>A COMPARISON OF HUMAN AND NON-HUMAN UREAPLASMAS.</u>	201
6.1	THE POLYPEPTIDE PATTERN AS SHOWN BY SDS-PAGE.	202
6.2	THE 96 kDa ANTIGEN.	204
6.3	THE 16 kDa AND 17 kDa ANTIGENS.	205
6.4	THE UREASE ENZYME.	210

6.4.1	POLYACRYLAMIDE GEL ELECTROPHORESIS.	210
<u>7</u>	<u>DIAGNOSTIC TESTS.</u>	220
7.1	FLUORESCENT TECHNIQUES.	221
7.1.1	INDIRECT METHOD.	221
7.1.2	DIRECT METHOD.	222
7.1.3	ANTI-96 kDa AND ANTI-16/17 kDa MABS.	222
7.1.4	SENSITIVITY OF THE FITC-CONJUGATED MABS.	227
7.1.5	ANTI-UREASE MABS.	230
7.2	THE UREASE CATCH ASSAY.	232
7.3	DIP-STICK TEST.	235
7.4	SLIDE AGGLUTINATION.	236
7.5	LATEX BEADS.	237
7.6	DYNOSPHERES.	240
<u>8</u>	<u>DISCUSSION.</u>	246
	<u>BIBLIOGRAPHY.</u>	264

CHAPTER 1
INTRODUCTION.

1.1. TAXONOMY.

The prokaryotes have been divided into four main divisions, based primarily on the nature of the bacterial cell wall (Murray, 1984). One of the major characteristics of mycoplasmas is their lack of a cell wall and thus these organisms have been placed in the division TERNICUTES, with the class designation MOLLICUTES, (Table 1). This designation was initially proposed by Edward and Freundt (1967), and complemented their original classification for wall-free organisms.

As can be seen from Table 1, the mycoplasmas have been divided into two orders: (1) MYCOPLASMATALES, those requiring sterols, and (2) ACHOLEPLASMATALES, those not requiring sterols. The MYCOPLASMATALES contains two families, the MYCOPLASMATACEAE and the SPIROPLASMATACEAE, with the ACHOLEPLASMATACEAE, as yet, the only family in the ACHOLEPLASMATALES. In addition, the ANAEROPLASMAS and the THERMOPLASMAS are Mollicutes of uncertain taxonomic position. However, the Thermoplasmas apparently belong to the Archaeobacteria (Maniloff, 1983; Rogers *et al.*, 1985).

Ureaplasmas, (formerly T-strain mycoplasmas), were first isolated by Shepard in 1954 and their existence was later confirmed by Ford *et al.* in 1962. In 1974, Shepard *et al.* proposed a new genus in the family MYCOPLASMATACEAE (UREAPLASMA) (Table 1), as these organisms were unique in their ability to hydrolyse urea.

Ureaplasmas have been divided into two species, *Ureaplasma diversum* and *Ureaplasma urealyticum*, isolated from bovine and human hosts respectively (Howard and Gourlay, 1982).

U. diversum was isolated from 33 % of urogenital tracts of cattle by Taylor-Robinson *et al.* in 1967 and again in 1969. At this time there was no direct evidence that these organisms caused disease as other large colony mycoplasmas and other bacteria were often present in the material being cultured. However, in many

TABLE 1: CLASSIFICATION OF THE MOLLICUTES.

Division: TERNICUTES, (wall-free prokaryotes).

Class: MOLLICUTES.

ORDER 1: MYCOPLASMATALES.

(i) sterol required for growth.

(ii) genome size $5 \times 10^8 - 1 \times 10^9$ daltons.

(iii) NADH oxidase located in the cytoplasm.

Family 1: MYCOPLASMATACEAE.

(i) genome size approximately 5×10^8 daltons.

Genus 1: MYCOPLASMA, (about 76 species)-

(i) do not hydrolyse urea.

(ii) some ferment glucose and/or hydrolyse arginine.

(iii) G + C of DNA from 22 - 41 mol%.

Genus 2: UREAPLASMA, (3 species).

(i) hydrolyse urea.

(ii) G + C of DNA from 27 - 30 mol%.

Family 2: SPIROPLASMATACEAE.

(i) genome size 1×10^9 daltons.

(ii) helical organisms during some phases of growth.

Genus 1: SPIROPLASMA, (5 species).

(i) ferment glucose and most hydrolyse arginine.

(ii) G + C of DNA from 25 – 31 mol%.

ORDER 2: ACHOLEPLASMATALES.

(i) sterol not required for growth.

(ii) genome size about 1×10^9 daltons.

(iii) NADH located in the membrane.

Family 1: ACHOLEPLASMATACEAE.

Genus 1: ACHOLEPLASMA, (about 10 species).

(i) G + C of DNA from 27 – 36 mol%.

MOLLICUTES OF UNCERTAIN TAXONOMIC POSITION:

Genus: ANAEROPLASMA, (2 species).

Genus: THERMOPLASMA, (now appears to belong to Archaeobacterium and has no specific relationship to other mycoplasmas (Rogers et al., 1985)).

cases they were isolated in large numbers (10^5 c.f.u. ml^{-1}). This organism was also isolated from pneumonic lungs of calves by Gourlay (1968) and these organisms were inoculated endobronchially into 16, three week old calves (Gourlay and Thomas, 1970). Of these 16 calves, pneumonic lesions were found in 14 and ureaplasmas were re-isolated from 13/16 lungs.

These organisms were recently inoculated into the amniotic cavity of four pregnant cows (Miller *et al.*, 1983); two of these cows suffered abortions and the other two produced calves, both with evidence of pneumonia, one of which died immediately after birth. It is now thought that these organisms also induce mastitis (Doig *et al.*, 1980), salpingitis and endometritis as well as spontaneous abortion and neonatal calf pneumonia (Gourlay *et al.*, 1972). *U. diversum* has also been shown to adsorb to both bovine embryos and ova, even after the washing procedures recommended for *in vitro* fertilisation have been carried out (Britton *et al.*, 1988).

At least twentyfive bovine strains have been studied and when examined by immunofluorescence and by one- and two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), can be divided into three groups, with group one being further sub divided into another three sub-groups (Howard *et al.*, 1975; Mouches *et al.*, 1981). Cluster A is represented by strain A417 (type strain), B by strain D48 and C by strain T44 (Barile, 1986). Although these strains are distinct, there is a continuous spectrum of antigens among the three clusters and strains from cluster A and B do not cross-protect on challenge (Barile, 1986). These strains show marked antigenic complexity and at least eight representative sera are required to identify all strains of *U. diversum* (Howard *et al.*, 1975), however, it has been reported more recently, (Howard and Gourlay, 1981) that bovine ureaplasmas can be subdivided into just the three type strains by antisera raised in gnotobiotic calves. These bovine strains appear to be very similar to those strains isolated from humans (*U. urealyticum*) in terms of growth requirements and also in terms of their short growth cycle and low titres compared with that of other large colony mycoplasmas

(Taylor-Robinson *et al*, 1971). However, these strains could be differentiated from each other using the metabolic inhibition test (Purcell *et al*, 1966) suggesting that strains from human and cattle are not antigenically identical. The G + C content of these organisms differed, when compared to ureaplasma isolated from humans (28.7-30.2 %, *U. diversum* (Howard *et al*, 1978), 27.7-28.5 %, *U. urealyticum*, (Bak and Black, 1968). Several strains isolated from cattle were subjected to SDS-PAGE and several distinct bands could be identified, although the protein profiles observed were very similar to those seen with human ureaplasmas, suggesting these strains, although not antigenically identical, may have many structural components in common (Taylor-Robinson *et al*, 1971; Howard *et al*, 1981).

Ureaplasmas have now been isolated from a number of animal species other than cattle. These include dogs, cats, nonhuman primates, sheep, goats, birds, pigs and mink (Howard and Gourlay, 1982), and both bovine and human ureaplasmas are apparently distinct from these ureaplasmas.

Based on SDS-PAGE and on immunofluorescence with antisera prepared in calves, the ureaplasmas isolated from sheep and goats can be regarded as comprising two serological groups not related to the species of origin (Howard and Pocock, 1983). However, the polypeptide pattern of these strains resemble those of *U. diversum*. Furthermore, the G + C contents of DNA from ovine-caprine ureaplasmas were similar to those of bovine strains (Howard *et al*, 1978). Thus it has been suggested that the ovine-caprine strains may represent additional subgroups of *U. diversum* (Howard and Pocock, 1983). Ureaplasmas isolated from non-human primates appear to form four serologic groups, as do the ureaplasmas isolated from canines. Like the ovine-caprine strains, the feline ureaplasma strains form two serogroups, whereas ureaplasmas isolated from birds form only one serogroup (Koshimizu *et al*, 1984; Barile, 1986). The avian ureaplasmas have been classified as the third species (*U. gallorale*), by Koshimizu (1987) on the basis of Metabolic Inhibition tests. As yet, this third species has not been recognised by the International Taxonomy Subcommittee.

Black and Krogsgaard-Jensen (1974) first separated human strains of *Ureaplasma urealyticum* into eight distinct serotypes using serological procedures (see next section). Subsequently this typing scheme was expanded to fourteen serotypes based on results from a modified Metabolic Inhibition test and a colony indirect epifluorescence test (Robertson and Stemke, 1982). Further study indicated that two separate groups could be distinguished based on DNA-DNA homology, (Christiansen *et al.*, 1981), restriction endonuclease DNA digestion patterns, (Razin *et al.*, 1983), polyacrylamide gel electrophoretic polypeptide profiles, (Mouches *et al.*, 1981) and sensitivity to manganese salts, (Robertson and Chen, 1984). Cluster A consists of serotypes 2,4,5,7,8,9,10,11,12 and cluster B consists of serotypes 1,3,6 and 14 but the results with serotype 13 were not definitive and as a consequence it has not been included in either cluster (Stemke and Robertson, 1985). However, more recently, sixteen serotypes have been identified using a complement-dependant mycoplasmacidal test (Lin, 1985).

1.1.1. SEROLOGY OF UREAPLASMA UREALYTICUM.

As yet, all human clinical isolates of ureaplasma belong to one species with 14 serotypes. As both symptomatic and asymptomatic individuals have proven culture-positive for ureaplasmas, the serological typing of clinical isolates may be an important approach in establishing the aetiological role of this organism in pathological conditions of man.

In the early 60's, due to the lack of serological tests, the number of serotypes within this group was unknown but there are now many different tests which can be utilised to serotype these organisms and each will be discussed in turn.

1.1.1.1. Metabolic Inhibition (M.I.) Test.

The Metabolic Inhibition test was first described by Jensen in 1964 for

mycoplasma species other than ureaplasmas. In 1966, Purcell *et al.* described such a test for ureaplasmas and suggested the presence of at least 6 serotypes. This test utilises the fact that ureaplasmas breakdown urea to ammonia which produces an increase in pH. This can be detected using a pH indicator, usually phenol red, and depends upon the inhibition of multiplication of the organism which is induced by specific antisera, usually with the addition of complement, with consequent delay in the rise in pH of the culture medium.

However, since the inhibitory activity of the antiserum is usually incomplete, the empirical use of the test depends upon reading the changes in pH at various times, usually when the control tube has risen by 0.5 of a pH unit. This control tube is then compared to tubes containing antiserum and complement. Thus, titres vary with the time when the readings are taken, the titres are often low and there are difficulties in the reproducibility of the test.

It has been suggested (Lin and Kass, 1970) that some of the variabilities are due to the inactivation of the fourth component of complement (C4) by ammonia. These workers suggested that if the concentration of urea was decreased, this would in turn decrease ammonia production and the subsequent inactivation of C4 and thus tests would be more reproducible. Using this method, these workers suggested the presence of at least 11 serotypes within this species (Lin *et al.*, 1972).

This test was modified in 1979 (Robertson and Stemke) by introducing a more reliable endpoint. This was facilitated by using bromothymol blue indicator broth. Again this system contained only a low percentage of urea (0.025 % compared with 0.05 % in the original test). These workers suggested the presence of at least 9 serotypes using this system and later identified another serotype (type 10) using this Modified Inhibition test and a colony indirect epifluorescence method (discussed later) (Robertson and Stemke, 1982).

Disadvantages of this test include the fact that mixed cultures cannot be identified

and fastidious organisms or slow growing serotypes may be wrongly typed.

1.1.1.2. Growth Inhibition (G.I.).

This test is similar to (1.1.1.1) above but it is performed on solid rather than in liquid media. Mycoplasma species can be identified by inhibition of growth with homologous hyperimmune serum. The serum was originally incorporated into the agar on which the mycoplasmas were subsequently plated (Edward and Fitzgerald, 1954) but this serological identification procedure was subsequently refined by saturating small filter paper discs with specific antisera and placing these discs on the agar surface (Clyde, 1964), and thus the volume of antisera required was greatly reduced. This test was further modified by Stanbridge and Hayflick (1967) by drying antibody-impregnated paper discs at 5° C and then storing them at -20° C where their inhibitory activity was retained for longer than 7 months.

However, ureaplasmas have been reported not to be inhibited by their homologous hyperimmune sera tested with the paper disc technique (Black, 1973a), but this could have been due to the lack of potent antisera. Several factors such as inoculum size and amount of antiserum are found to influence this test. Black (1973a) suggested the use of antisera in wells rather than on discs as this increased the zone of inhibition due to the increase in diffusion through the agar. The incubation temperature was also critical and was found to be optimal at 27°C as this decreased growth rates and allowed sufficient diffusion of antisera. Using this test, 8 serotypes were identified (Black, 1973a).

The major advantages of the Growth Inhibition test are its simplicity and specificity (Black, 1973a; Piot, 1977); however, this test is less sensitive than indirect immunofluorescence (see later) and the Metabolic Inhibition test. Mixed cultures are also difficult to identify and a cross reaction is often seen with serotype 2 and serotype 5 (Piot, 1977).

1.1.1.3. Immunofluorescence.

This method of classification involves the growth of the mycoplasma on solid agar plates. The colonies are cut from the agar, placed on slides and can be either stained without prior fixation or fixed at 80°C in distilled water. However, hot water fixation of ureaplasmas tends to increase the number of cross reactions seen (Black and Krosgaard-Jensen, 1974). In addition, the fluorescence of unfixed colonies is uniform, whereas hot water-fixed colonies tend to fluoresce in the centre of the colony only (Rosendal and Black, 1972).

Both indirect and direct staining methods have been utilised with varying success. Indirect immunofluorescence (indirect epifluorescence) was first used for serological classification of ureaplasmas in 1970 by Black and again in 1974 by Black and Krosgaard-Jensen. This method appears to produce less background fluorescence compared to the direct method but this could be due to the specificity and/or the fluorochrome to protein (F/P) ratio of the labelled sera. The indirect method appears to be more sensitive than the direct method according to these workers and utilising this technique, 8 serotypes were identified with one cross-reaction between antiserum to serotype 2 and serotype 5 antigen.

This technique was later modified by Stemke and Robertson in 1981, such that colonies were cut from plates using a cylindrical punch and transferred to a 96-well plate. Thus the procedure is faster and less cumbersome and consequently large numbers of isolates can be processed together. Plot (1977) using the indirect method, reported several cross-reactions:

antiserum against type 2 with antigen types 1 and 5.

antiserum against type 4 with antigen 2.

antiserum against type 8 with antigen types 4 and 7.

However, if colonies were washed overnight after staining, this reduced background and some weaker cross-reactions. Results from this test are also subjective to read.

This method has two advantages, it can be applied to primary growth on agar and can be used to identify mixed serotypes. Unfortunately, stained and unstained colonies cannot be viewed simultaneously.

1.1.1.4. Indirect Immunoperoxidase.

This test is an alternative to the immunofluorescence test and was described by Polak-Vogelzang *et al.* in 1978 with respect to *Acholeplasma* and *Mycoplasma* as many of these colonies autofluoresce.

This test is carried out as for the indirect immunofluorescence test with the secondary antibody being tagged with peroxidase rather than a fluorochrome. The result is seen by adding 3,3'-diaminobenzidine tetrahydrochloride solution and hydrogen peroxide since this substrate forms a dark brown precipitate on the colonies in the presence of the enzyme. In most cases this test is as sensitive as the immunofluorescence test.

This method was used in 1981 (Quinn *et al.*) for the identification of *U. urealyticum* serotypes and was reported as being ideal for detecting and identifying mixed serotypes as stained and unstained colonies could be visualised simultaneously. Highly dilute antisera can also be used which removes any cross-reactivity which is sometimes seen with both the Growth Inhibition and immunofluorescent tests.

Thus, this test is not only simple and rapid, but can be done directly on primary plates and is also sensitive and specific.

One disadvantage is the variation in the intensity of staining from day to day, also colonies of bacteria or yeast occasionally stain due to endogenous peroxidase

activity.

This technique was modified by Imada *et al.* in 1987 such that antiserum was adsorbed to squares of filter paper and these were applied to individual colonies. Similarly, filter paper saturated with conjugate, was applied to the colony and developing solution was then poured on to the agar plate surface. This modification allows one plate to be tested with several antisera without having to transfer agar blocks to another vessel. However, occasionally colonies peel off the plate during the staining procedure and this results in poor staining. This test has recently been used to detect an antibody response in patients sera and appears to be very sensitive (Quinn and Th'Ng, 1988).

1.1.1.5. DNA Homology.

Many of the pitfalls of traditional taxonomic methods can be circumvented by directly comparing the genetic constitution of organisms. G + C content and DNA hybridisation methods were used by McGee *et al.* (1967) to study relationships among Mycoplasmas, L-forms and bacteria. These workers concluded that there was extensive genetic heterogeneity amongst mycoplasmas and suggested that L-forms were not related in any way to mycoplasmas.

In 1968, Bak and Black, studied the DNA base composition of 7 ureaplasma strains using thermal denaturation (T_m) and G + C contents were determined and found to be 27.7-28.5 % but no significant difference was found between individual serotypes.

In 1969, Bak *et al.* studied genome size of several ureaplasmas and other mycoplasma species including 7 strains requiring sterols and 3 strains not requiring sterols (Acholeplasmas). These studies suggested that those strains not requiring sterols constitute a distinct group with a genome size of 1.0×10^9 daltons and sterol-requiring organisms have a genome size of about 4.6×10^8 daltons.

The above results were confirmed by Black *et al.* (1972b) using Tm and CsCl gradient centrifugation suggesting the correct classification of the ureaplasmas with the mycoplasmas, and the inability of these techniques to serotype these organisms.

1.1.1.6. Enzyme Linked Immunosorbent Assay (ELISA).

Eight serotypes of *U. urealyticum* were studied and characterised using a modified ELISA with a sonicated antigen suspension (Turunen *et al.*, 1982). These workers noted cross-reactions with serotypes 2 and 5 and also with serotypes 4,7 and 8 and suggested this was due to the sharing of a common antigenic component. These workers also claimed the ability to detect mixed cultures with this test.

This test was also studied by Wiley and Quinn (1984) for the detection of specific antibodies to *U. urealyticum* serotypes, where they compared both soluble and particulate antigens. The soluble antigen was apparently more sensitive suggesting that purified antigen may not be necessary to obtain serotype specificity. Again, a one-way cross reaction was seen with type 2 antisera and type 5 antigen.

In contrast, Brown *et al.* (1983) found their ELISA method gave nonspecific results for the 8 serotypes. However, the antigen was prepared from organisms lysed with 0.05 M carbonate-bicarbonate buffer, pH 10.0, but this should not account for the difference in specificity. Wiley and Quinn suggested the difference may be due to the standardisation of the ELISA method used.

This method when standardised correctly appears to be rapid, simple, reproducible and economical with reagents.

1.1.1.7. Isoelectric Focussing and Polyacrylamide Gel Electrophoresis (PAGE).

Electrophoretic patterns of the cell proteins of 12 ureaplasmas isolated from man were studied (Razin *et al.*, 1970). The results observed suggested that these 12 strains

represented a group of closely related strains possibly belonging to the same species. As horse serum from the medium was found to co-pellet with the organisms, this was replaced with 1 % (v/v) PPLO serum fraction which appeared to alleviate this problem.

Black and Krossgaard-Jensen (1974) confirmed the above results when they studied 8 serotypes and these workers felt that this method was unsuitable for the differentiation between, and identification of, the serotypes.

Sayed and Kenny (1980) identified 36-40 polypeptides and suggested that at least 80 % of these were common among the 8 serotypes studied; this was confirmed by isoelectric focussing. To prevent medium contamination of cell pellets these workers used a dialysate broth base supplemented with agamma serum.

Howard *et al.* (1981) modified this technique such that PAGE was performed with polypeptides labelled by growing organisms in the presence of ^{35}S -labelled methionine. These workers compared bovine and human isolates and found the bovine strains had many common peptides, as did the human isolates. The human isolates could however be distinguished from the bovine isolates, thus supporting the view that bovine ureaplasmas should be regarded as a separate species from *U. urealyticum*.

Mouches *et al.* (1981) also studied 12 human serotypes using this modified technique. Both 1- and 2-dimensional PAGE were utilised and these workers differentiated the human ureaplasmas into 2 distinct groups, a finding confirmed by Swenson (1983) who studied 9 serotypes.

Labelling whole cells with ^{35}S -labelled methionine avoids the problems of contamination with growth medium, but some of the polypeptides may fail to incorporate this amino acid.

1.1.1.8. Immunobinding.

This method has been described by Kotani and McGarrity (1985) and is a modification of that described by Hawkes *et al.* (1982). Briefly, 10 µl of test specimen, cell culture supernatant, broth culture or clinical sample are dotted onto nitrocellulose paper which was previously washed in distilled water. This is followed by the application of specific anti-mycoplasma antisera and after incubation, an enzyme conjugated antiserum against the first antisera is applied. A positive reaction is indicated by the development of an intense blue colour reaction when substrate is applied.

Nonspecific reactions are seen with this test but can be eliminated by further diluting the primary antisera. However, this test requires a cell concentration of at least 10^4 c.c.u. ml⁻¹ but has the advantage of being rapid and simple as well as not having the need for the growth of the organisms providing they are present in high enough numbers in the clinical sample. This could be a problem for mycoplasmas such as ureaplasmas.

1.1.1.9. Indirect Haemagglutination Test.

This test was described by Stavinsky (1954) and was used successfully for the detection of *Mycoplasma pneumoniae* antibody by Dowdle and Robinson (1964). Seven human ureaplasmas were also typed using this method (Black, 1970), although high homologous titres were seen for each of the seven serotypes, various degrees of cross-reactivity occurred. Krosgaard-Jensen (1971) also reported the successful use of this test for typing both mycoplasmas and ureaplasmas.

1.1.1.10. Direct Test For Urease.

Ureaplasmas grown on solid media can be differentiated from other mycoplasmas

using this test (Shepard and Howard, 1970).

Ureaplasma colonies are first exposed to urea and the ammonia produced is detected by manganous chloride (0.8 % w/v) since deposits of manganese dioxide are formed on the surface of the colonies. Colonies must be young as the test may be unreliable if applied to ureaplasma colonies that have been incubated longer than 48 hr at 37°C. However, this test will not differentiate serotypes of ureaplasmas.

The above tests have divided *U. urealyticum* into at least 14 serotypes (Robertson and Stemke, 1982) although more recently, 16 serotypes have been putatively identified using the Modified Metabolic Inhibition test (Lin, 1985.; Kass *et al*, 1986).

The ideal method for serotyping strains of ureaplasmas should have a high specificity and sensitivity with economical consumption of antisera and other reagents. The method should also be simple and rapid in order to be suitable for typing a large number of organisms and the results should be reproducible.

Many of the above tests have limitations due to complex technical procedures and difficulties in standardising the test results and thus a combination of at least two methods is usually used to confirm results. This is time consuming and expensive and many of the above techniques can only be carried out by laboratories with access to appropriate antisera/antigens. Recently, antisera to the 14 serotypes of *U. urealyticum* have been prepared (Th'Ng and Quinn, 1988) and are available through the Research Development Corporation at the Hospital for Sick Children, Toronto, Canada.

1.2. CELL MORPHOLOGY AND SIZE.

The most important characteristic that distinguishes the mycoplasmas from other prokaryotes is their total lack of a cell wall and consequently, mycoplasmas are sensitive to lysis by osmotic shock, alcohols, organic solvents and detergents. This

lack of a cell wall also accounts for the plasticity of these organisms, and as is to be expected from plastic organisms, the coccus is the basic form of all mycoplasmas. However, elongated, fusiform, bottle-shaped or filamentous forms of some species can be observed under different growth conditions (Reviewed by Maniloff and Morowitz, 1972).

Due to the different methods used, there has been wide disagreement on the minimal size of mycoplasmas (Reviewed by Razin, 1969). Studies using filtration methods usually do not specify the positive or negative pressure applied and this is important as the plastic organisms can change their shape and squeeze through filter pores smaller than their actual diameter (Morowitz, 1967). Cells of several mycoplasmas were found to pass through Millipore filters of 0.22 μm pore diameter when the Swinney hypodermic adapter was used. The positive pressure, which is apparently quite considerable, cannot be controlled using this method (Razin, 1969) but at a controlled negative or positive pressure, many mycoplasma cells did not even pass through 0.45 μm pore diameter filters (Razin *et al.*, 1968).

Electron microscopy has been frequently used to measure the diameter of sectioned cells. Membrane-bound bodies filled with ribosomes and having a diameter of 0.1–0.2 μ have been observed and these were considered to be the minimal reproductive units (Anderson and Barile, 1965). However, the values obtained by measuring sectioned cells may vary considerably with the plane of the section and could be influenced by the method of fixation employed.

There is now general agreement amongst mycoplasmologists that the diameter of individual mycoplasmas varies from 0.33–1.0 μm and these represent the smallest reported living cells (Morowitz, 1967; Razin, 1978). Observations by Robertson *et al.* (1975) support the above conclusion by showing that the small spherical bodies, 0.1–0.25 μm in diameter, isolated from *Mycoplasma hominis* cultures could not reproduce.

Ureaplasmas are generally round to ovoid and are reported as being between 0.3-0.8 μm in diameter, (Razin *et al.*, 1977) and have a colony size of 7-20 μm (Shepard, 1954), but Manchee and Taylor-Robinson (1969) reported colonies of greater than 200 μm in diameter with the addition of HEPES buffer. Black *et al.* (1972) have also reported the observation of short rod-shaped cells and filamentous partially branched forms. Nevertheless, the majority of ureaplasmas are single, ovoid cells and filaments or organisms attached in long chains were not found by Razin *et al.* (1977).

1.2.1. THE CELL MEMBRANE OF UREAPLASMAS.

The cells are enclosed by a single tri-laminar membrane approximately 10 nm thick but a ruthenium red layer appears on the surface of the membrane and is approximately 20-30 nm thick. Application of Concanavilin A-iron dextran stain indicates that this layer contains glucosyl-like residues (Robertson and Smook, 1976). Whilst this layer may represent an adsorbed accumulation of carbohydrate from the growth medium, different strains of ureaplasmas differ in their ability to produce a ruthenium red-positive capsule even when grown in the same conditions and hence it may represent a lipoglycan synthesised by the organism because of its resemblance to the galactan from *M. mycoides*. Lipoglycans are polysaccharides covalently attached to lipids and are found as surface structures on many Mollicutes. Smith (1985) has examined serotypes 3,4 and 8 of *U. urealyticum* and found lipoglycans associated with all three and showed that all the sugar components were neutral in character and consisted of mannose, glucose and galactose. Glycerol, fatty acids and phosphorus were all integral components of these structures and all three serotypes exhibited ratios of components and neutral sugars distinct from each other. Lipoglycans are thought to play a role in maintaining structural stability and/or in adhesion of the organism to mucosal surfaces (see section 1.7.4).

No surface appendages such as flagella have been observed but it has been reported by Black *et al.* (1972a) that ureaplasma cells have an electron dense layer

consisting of hair-like structures. They have suggested that these hair-like structures play a role in reinforcing the membrane. They may also play a role in adherence of the organism to the epithelial cells of the urinogenital tract or they may be involved in haemadsorption, but since only serotype 3 adsorbs to erythrocytes, this explanation seems unlikely (Black, 1973b).

Unlike the situation with many walled bacteria, the membrane is easily isolated by gentle procedures such as osmotic shock. The maximum osmotic pressure tolerated by most mycoplasmas lies in the range of 12-14 atms (Leach, 1962) and the organism's sensitivity to osmotic shock apparently decreases with the increase in the age of the culture (Razin, 1964), and with the presence of even trace amounts of divalent cations in the growth medium (Rodwell, 1965). Changes in the osmotic fragility of the cells can be caused by variations in the cholesterol content or in the ratio of unsaturated to saturated fatty acids in membrane lipids and these parameters apparently alter with ageing of the culture (Razin, 1979a).

Other methods for isolating sterol-requiring mycoplasma membranes include digitonin-induced lysis since digitonin forms a complex with cholesterol in the membrane, apparently causing considerable rearrangements in the lipid bilayer, leading to an increased permeability and cell lysis (Rottem and Razin, 1972). The success of this technique is not influenced by culture age or the presence of divalent cations but the membranes seem to retain the cholesterol-digitonin complex so that their organisation and composition are altered.

Alkali lysis, alternate freezing and thawing and sonication are other methods also used. Romano and LaLicata (1978) showed that ultrasonic treatment disrupted all the cells in a cell suspension of ureaplasmas, whereas osmotic shock only lysed about 50% of the cells and digitonin only about 70 %. Like other biological membranes, mycoplasma membranes consist essentially of proteins (50-60%) and lipids (30-40%). However, these membranes are often contaminated with precipitated components of

the growth medium and this seriously hinders their chemical, enzymatic and antigenic characterisation. As ureaplasmas are routinely grown at a low pH, (pH 6.0) this may cause precipitation of proteins and lipoproteins from the serum component of the growth medium, which then co-sediment with the cells during harvesting. The cell yield of ureaplasmas is so low that most of the pellet may consist of non-cellular components. Masover *et al* (1976), reported the protein content of the 'membrane' fraction isolated by digitonin treatment of *U. urealyticum* grown in unfiltered Hayflick's medium, was about 25 times higher than the amount in the cytoplasmic fraction. Reduction of the serum content and pre-filtration of the growth medium reduced the membrane to cytoplasmic protein ratio to approximately 10 (Masover *et al*, 1977b). Centrifugation on sucrose gradients also separates the ureaplasma cells from nonspecific precipitates. Thus, analysis of ureaplasma membrane preparations as reported by Whitescarver *et al*. (1975) are clearly questionable.

As mentioned previously, ureaplasmas require fatty acids and sterols (usually in the form of cholesterol) to support growth. Cholesterol makes up approximately 12-30% of the total membrane lipid and free fatty acids and phosphatidic acid are the other predominant lipids of ureaplasmas (Romano *et al*, 1972) with the remaining neutral lipids being composed of cholesterol esters, triglycerides and diglycerides. Three glucose-containing glycolipids are also present as well as several other phospholipids (Rottem *et al*, 1971). Crude estimates of the lipid composition of ureaplasmas suggest a ratio of neutral to phospholipids of about 1:1 and of cholesterol esters to cholesterol of about 1:4.

In ureaplasmas, free fatty acids have been postulated to control the function and integrity of the membrane by neutralisation of the copious quantities of ammonia produced by urea hydrolysis. The production of strongly acidic lipids, such as phosphatidic acid, may also help to compensate for this production of ammonia (Romano *et al*, 1972).

The only common feature of the polar lipids among all members of the Mollicutes is the existence of acidic glycerophospholipids (Smith, 1973), phosphatidyl glycerol and diphosphatidyl glycerol are ubiquitous. Unique polar lipids are found in *Acholeplasma*, *Thermoplasma* and *Ureaplasma*. *Ureaplasmas* are the only member of the Mollicutes yet shown to contain the predominant bacterial phospholipid, phosphatidyl ethanolamine (Romano *et al.*, 1972) and in addition to this positively charged lipid, *ureaplasmas* also contain an unidentified amino lipid which appears to be a diamino hydroxy compound containing adjacent fatty acid esters and N-acyl groups. The uniqueness of the lipids of *ureaplasmas* may reflect both its acidic pH optimum for growth and its copious ammonia production.

Carbohydrate appears to be a minor component (0.5–2% dry weight) of any mycoplasma membranes as yet analysed. Studies on mycoplasma membranes have suggested that the lipids are organised as a bilayer with the carbohydrate containing lipids preferentially located in the outer half of the bilayer whilst phospholipids and cholesterol in mycoplasmas are present in both sides of the membrane but often in different proportions.

Most mycoplasma membrane preparations also contain a small amount of RNA and DNA which are presumably cytoplasmic contaminants as they can be removed by washing and nuclease treatment (Razin, 1969).

1.3. REPRODUCTION.

The dispute over the morphology of mycoplasmas is part of the controversy surrounding their mode of reproduction. It is still impossible to follow the reproduction of a single *ureaplasma* cell because of its minute dimensions and descriptions are therefore based on representative series of sequential electron micrographs for a number of individual dividing cells. The theory that reproduction takes place through the liberation of many elementary particles by large bodies

(Klieneberger-Nobel, 1962) seems to be loosing ground, as larger bodies are coming to be recognised as swollen, mostly dead cells. The mycelial theory, involving the development of elementary bodies into filaments which subsequently break up to form new elementary bodies has received strong support from phase-contrast microscopy studies (Razin and Cosenza, 1966).

Several workers have suggested that mycoplasmas reproduce by budding, whereas more recently, others have suggested reproduction occurs by binary fission. For typical binary fission to occur, cytoplasmic division must be fully synchronised with genome replication, and in mycoplasmas, cell septation may lag behind genome replication, resulting in the formation of multinucleate filaments, (Reviewed by Razin, 1969, 1978; Maniloff and Morowitz, 1972).

1.4. CELL GROWTH

The small genome size and restricted metabolic capabilities (see section 1.8) explain the fastidiousness of mycoplasmas in their cultural requirements. Indeed, there may be species of mycoplasmas which remain to be discovered due to the lack of an appropriate medium for their isolation and growth.

Mycoplasmas are sensitive to environmental conditions such as the tonicity of the medium, are nutritionally exacting and require a large array of precursors for the synthesis of macromolecules. Many of the complex media that have been described for the cultivation of mycoplasmas are based on that of Edwards (1947) and usually contain peptone, beef heart infusion, yeast extract to supply nucleic acid precursors and other growth factors, sodium chloride to adjust tonicity and animal serum. The serum provides fatty acids and cholesterol in an assimilable and non-toxic form for membrane synthesis, and the albumin component of serum functions as a carrier and detoxifier of the fatty acids.

Unfortunately, the presence of animal serum usually from the horse, creates

difficulties when harvesting the cells as many of the serum components adsorb to the surface of the organisms and consequently co-pellet with the cells thus contaminating the preparations.

Attempts to replace serum with better defined or less complex materials have only been partially successful, usually resulting in very low titres of cells.

The ureaplasmas are distinguished from all other Mollicutes by possession of the enzyme urease (Shepard and Luncford, 1967). Most evidence suggests that urea is an essential substrate for growth, although earlier, Masover *et al.* (1973; 1974) had suggested that urea was not essential for growth and could be substituted with allantoin and/or putrescine dihydrochloride. However, it is possible that trace amounts of urea were present in the serum component of the medium. The fact that specific urease inhibitors inhibit the growth of ureaplasmas (Kenny, 1983), supports the key role of urease in ureaplasma growth and it has been suggested (Romano *et al.*, 1986) that this enzyme is involved in energy production (section 1.5).

Most ureaplasma media are supplemented with small amounts of urea (0.05%) since higher concentrations (0.25 M) are inhibitory to the cells and the presence of the urease does not appear to protect these organisms when compared to *Mycoplasma hominis* and *Acholeplasma laidlawii* (Masover *et al.*, 1977a). The urea is metabolised to carbon dioxide (CO₂) and ammonia and the production of the latter causes a rise in pH which can be detected by an indicator (eg, phenol red, bromothymol blue) and in liquid culture, this change in colour is used to detect the growth of the organisms.

Growth of ureaplasma cells occurs optimally at a starting pH of 6.0 and the pH of the medium rises to approximately pH 7.9 at the stationary phase of growth (see Fig 1.1).

Solid media for ureaplasmas are of two types: differential agar medium and standard agar medium. Differential agar medium (A7), which is available commercially,

includes a divalent cation indicator such as manganous sulphate which detects the production of ammonia and consequently, urease activity (Shepard and Lunceford, 1976). Shepard and Coombs (1979) modified this medium by adding putrescine which increased the size of the colonies seen and this modified medium is known as A7B. Growth of ureaplasma colonies on standard agar medium can be detected by the spot test which involves the addition of manganous chloride on to suspected ureaplasma colonies (Shepard and Howard, 1970; see section 1.1.1.10); the best growth of ureaplasmas on solid media is in an atmosphere of 5% CO₂ in nitrogen.

Characteristically, ureaplasmas do not grow to titres higher than 10⁸ colour changing units (ccu) (see section 2.2) *in vitro*. When ccu's are assayed, they follow typical bacterial growth curves, (Fig 1.1), with lag, exponential, stationary and death phases. In the exponential phase at 37°C, most cultures have a doubling time of one to six hours. The length of the stationary phase varies but is frequently very short, around 6h (Maniloff and Morowitz, 1972), or if the medium is buffered with (a) CO₂, 27h, but with no increase in viable cell numbers (Thirkell, personal communication, 1987), or (b), with HEPES (Manchee and Taylor-Robinson, 1969). The subsequent death rates are also quite rapid; the titre drops by a factor of 10² - 10⁴ c.c.u. h⁻¹, even in well buffered medium (Kenny and Cartwright, 1977; Masover et al., 1977a).

Whether this rapid decline is due to the rise in pH caused by the production of ammonia is uncertain. As previously mentioned, bubbling CO₂ through the medium does lower the pH but does not improve the growth of these organisms, thus some other factor must be involved (Masover *et al.*, 1977a).

Exhaustion of urea or other medium components is also apparently not the cause, and Furness (1973) has suggested the presence of a catalase-resistant, heat-stable, dialysable toxic product which apparently inhibited all strains of ureaplasmas tested.

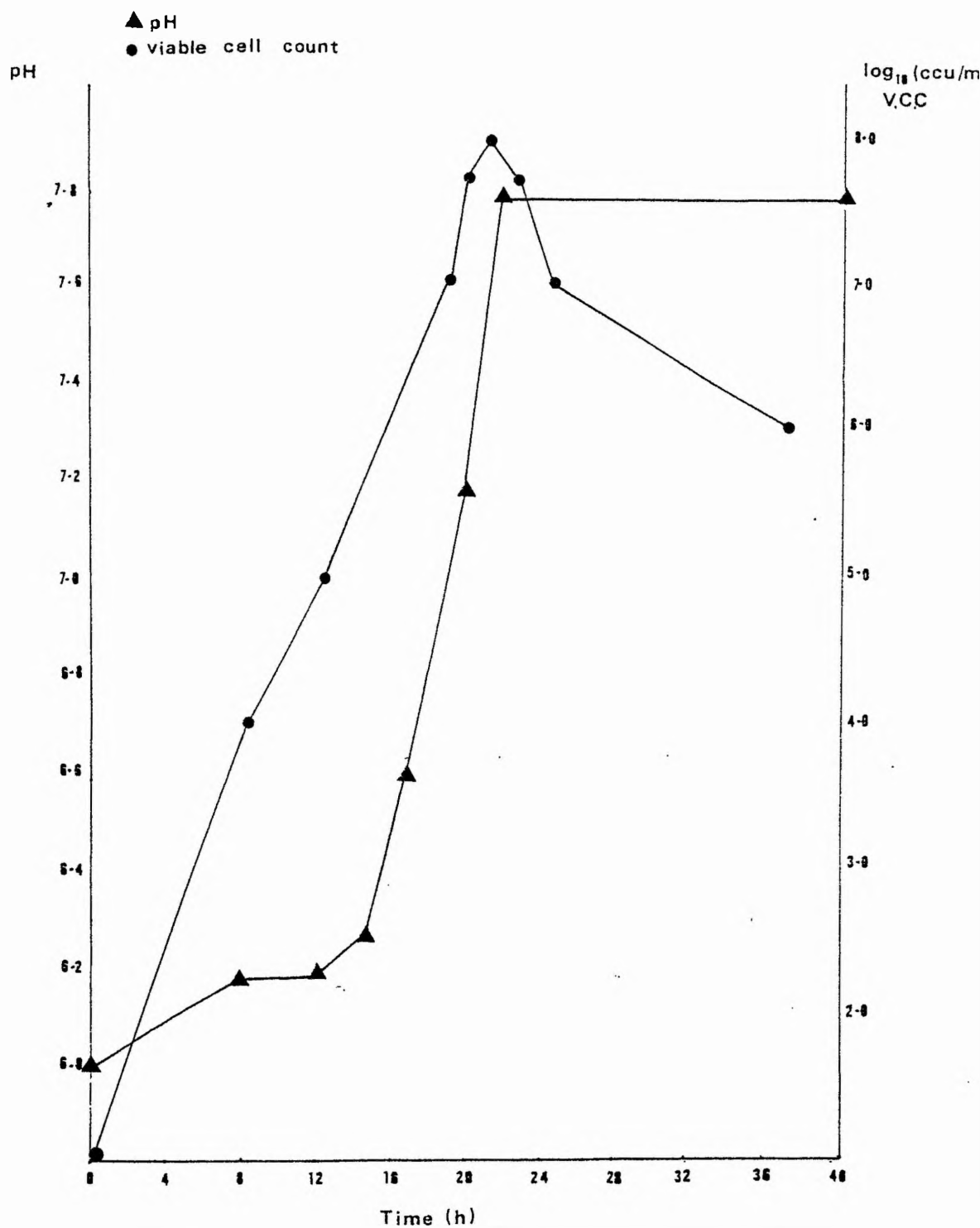


Fig 1.1: Growth and pH profile of 10 μl seed (10^5 c.c.u. ml^{-1} of *U. urealyticum* 100 ml^{-1}).

1.5. METABOLISM

The metabolism of ureaplasmas is poorly defined with low cell yields still the major factor limiting biochemical studies. *U. urealyticum* appears to lack the conventional mechanisms for adenosine-5-triphosphate (ATP) generation such as glycolysis or arginine breakdown, present in other mycoplasmas. Many of the enzymes which have been shown to be present and absent have been discussed by Cocks *et al.* (1985) and by Pollack (1986). The inability to detect enzymes in ureaplasmas may reflect the poor cell yields rather than the true absence of the enzymes, and Cocks *et al.* (1985) found that using sensitive radioactive assays helped to overcome this problem.

Using these assays, Cocks *et al.* (1985) have detected the activity of certain enzymes in the latter half of the glycolytic pathway (phosphofructokinase, aldolase, triose-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase, malate dehydrogenase, enolase and pyruvate kinase), but the activities observed for the glycolytic enzymes in extracts from *U. urealyticum* were considerably lower than those in *M. mycoides* (Cocks *et al.*, 1985). This could have been due to dilution of the small amount of *U. urealyticum* protein in the extracts by contaminating protein from the medium (see section 1.2.1). The extent of the differences between the two activities suggests a different role of the glycolytic pathway in these two organisms, i.e. that glycolysis is not a major energy producing pathway in *U. urealyticum*. Nevertheless, the presence of many glycolytic enzymes does suggest some role for them in carbohydrate metabolism (Cocks *et al.*, 1985). The lack of hexokinase and phosphoglucose isomerase activity in *U. urealyticum* (Cocks *et al.*, 1985; Pollack, 1986) is in keeping with the inability of this organism to ferment glucose. This may suggest that substrates other than glucose are feeding this sequence of reactions and generating phosphoenol-pyruvate and pyruvate. Contrary to this, O'Brien *et al.* (1983) detected phosphoglucose isomerase and lactate

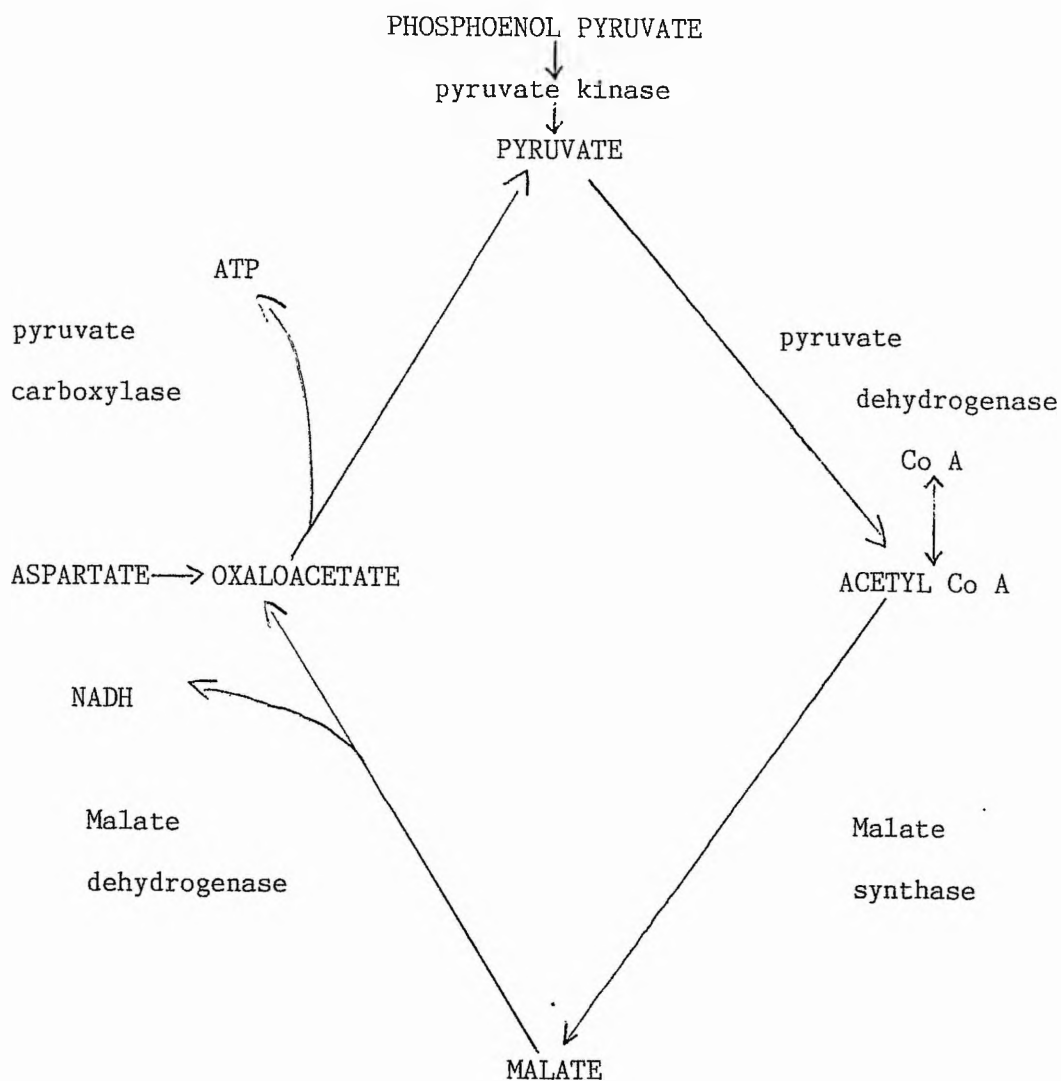


Fig 1.2: Metabolism around the pyruvate locus in *Ureaplasma urealyticum* (as suggested by Davis et al.,1988).

dehydrogenase in serotypes 1 and 2 of *U. urealyticum*.

Davis *et al.* (1988), have also detected the activities of pyruvate kinase and malate dehydrogenase as well as pyruvate dehydrogenase, pyruvate carboxylase, malate synthase and aspartate aminotransferase (Fig 1.2) and the presence of these enzymes may suggest that the carbon of pyruvate can be recycled, passing to malate, oxaloacetate and then back to pyruvate, or that pyruvate may be used in anabolic pathways. Thus, although *U. urealyticum* is apparently a metabolic degenerate organism which cannot catabolise sugars through a complete glycolytic-TCA pathway, pyruvate may still function as a pivotal intermediate in the metabolism of this organism.

In addition, *U. urealyticum* has most of the enzymes for pentose phosphate metabolism and this may indicate a capability for the formation of ribose-5-phosphate for nucleotide synthesis from glycolytic intermediates, or these enzymes could be part of a catabolic pathway from nucleoside to glycolytic intermediates. However, the role of this pathway in ureaplasma metabolism is still not clear (Cocks *et al.*, 1985). *U. urealyticum*, also has enzymes for the degradation of nucleotides and nucleosides, and for further metabolism of the ribose and deoxyribose phosphates produced. The enzyme adenylate kinase, which had comparatively high activity, was found in extracts from *U. urealyticum*. The significance of a capacity to rapidly interconvert AMP, ADP and ATP in this organism also remains unclear (Cocks *et al.*, 1985).

The enzymes inosine nucleosidase, adenine deaminase, adenosine phosphorylase and possibly inosine phosphorylase, and AMP nucleosidase, have been detected in ureaplasma cell lysates and so this organism can metabolically generate hypoxanthine from AMP, adenosine, inosine and adenine. The formation of hypoxanthine suggests a possibility for the existence of a purine salvage pathway (Davis *et al.*, 1984). When these pathways are operational, adenine, hypoxanthine, and/or guanine, can be recycled by using them for mononucleotide synthesis. *U. urealyticum* is

non-fermentative, (Shepard and Masover, 1979) and no energy source for its growth have been defined with the possible exception of urea.

However, urea carbon is not incorporated into cellular material as most of it is excreted as CO_2 (Ford *et al.*, 1970). Thus, although it is unknown where ureaplasmas acquire their carbon for cell mass, one possibility arises from the observations of Delisle (1977) who also found malate dehydrogenase activity and α -glycerophosphate dehydrogenase activity, again suggesting that there is other enzymatic activity near the pyruvate locus, (see earlier), perhaps involving the tricarboxylic cycle.

Since urease is thought to be cytosolic, (Vinther, 1976; Masover *et al.*, 1977b), urea hydrolysis would be expected to release ammonia in the cytoplasm. Masover *et al.* (1977b), have suggested that the ammonia will take up protons at a physiological pH to become ammonium ions. The intra-cellular NH_4^+ could then play a role in proton elimination or acid-base balance which may be coupled to an energy producing ion gradient and/or transport mechanisms.

More recently, Romano *et al.* (1986) have shown that ATP is produced when urea is added to resting ureaplasma cells and its formation requires both the concomitant activity of the cytoplasmic urease and membrane-bound ATPase. ATPase is found in the cell membranes of most mycoplasmas; however, attempts to release this protein from membranes have as yet failed. Studies have suggested that the mycoplasma ATPase is an integral membrane protein probably dependent on lipids for activity (Razin, 1979b). Romano *et al.* (1986) have suggested that this energetic process depends upon phosphate, urea, pH and ammonium ions in the reaction mixture, but ammonium ions appear to interfere with the production of energy when the starting pH is slightly basic, perhaps explaining why ureaplasma, unlike other mycoplasmas grow best at a starting pH of 6.0.

Masover *et al.* (1977b), and Pollack (1986), have been unable to detect oxygen-dependent NADH oxidase activity and have suggested that the oxidation of

NADH requires an acceptor other than oxygen. If the absence of oxygen-dependent NADH activity is confirmed, then this finding would further distinguish the ureaplasmas from the other Mollicutes.

Inorganic pyrophosphate (PPi) and the roles of inorganic pyrophosphatases (PPase) in microbes have been reviewed by Lahti (1983). PPi may be a substrate functioning as a source of energy while donating orthophosphate in ATP-sparing reactions.

Davis *et al.* (1987) found PPase activity in *U. urealyticum*, serotypes 8 and 6, and that PPase activity is present in organisms with such a small genome may indicate that PPi hydrolysis is a basic activity in the cell. The presence of PPi-dependent phosphofructokinase in *Acholeplasma laidlawii* suggests that PPi utilisation in this organism is an energy conserving process (Pollack and Williams, 1986).

Romano *et al.* (1980; 1986) have suggested that orthophosphate concentration influences urea hydrolysis which in turn affects ATP synthesis and it may be that PPase helps to regulate phosphate levels in these organisms thereby optimising urease activity and subsequent ATP synthesis (Davis *et al.*, 1987).

1.6. PATHOGENICITY

There are at least 90 different species of Mollicutes now recognised, of which more than one third are pathogenic to man, other mammals, birds, insects or plants. Of these 90 species, 12 have been isolated from humans (Embree and Embil, 1980) and nine of these have been found in the respiratory tract and seven in the genitourinary tract, but only *Mycoplasma pneumoniae*, *Mycoplasma hominis*, *Ureaplasma urealyticum* and possibly *Mycoplasma genitalium* are thought to be pathogenic. To establish a causal relationship between mycoplasma infection and disease it is necessary to demonstrate the following:-

- (1) the organisms are isolated more frequently and /or in large

numbers from patients with disease than from those without.

- (2) antibody responses, measured by various techniques (see earlier), occur in patients with disease.
- (3) treatment with effective antibiotics leads to the disappearance of organisms and symptoms.
- (4) the organisms infect an animal host from which they can be recovered and, in doing so, produce a disease similar to that seen in man. The major problem in this respect with *U. urealyticum* is that the chimpanzee is the only good animal model and this has restricted the number of studies carried out.

This section on pathogenicity will be restricted to the claimed involvement of *U. urealyticum* in human disease processes, although *M. hominis* is frequently co-isolated with this organism.

The major disease states with which *U. urealyticum* has been implicated are as follows:-

1.6.1. NON GONOCOCCAL URETHRITIS (N.G.U.)

N.G.U. is diagnosed when urethritis is present in the absence of *Neisseria gonorrhoeae* and numerous causative agents have been suggested (Bowie, 1984; Reviewed by Taylor-Robinson, 1983). *U. urealyticum* and/or *Chlamydia trachomatis* have been isolated from the majority of N.G.U. cases (70-80%) and *C. trachomatis* itself is thought to be the causative agent of 40-50% of such cases (Taylor-Robinson and McCormack, 1980). Most studies initiated before 1973 did not include an investigation for this organism and thus these studies are difficult to interpret and provide conflicting data.

Ureaplasmas were first shown to be associated with this condition by Shepard (1954) who isolated this organism from urethral discharge and this was soon confirmed by Ford *et al.* (1962).

However, it is still unknown what proportion of N.G.U. cases are due to

ureaplasmas but the fact that it is responsible for some cases is no longer disputed (Taylor-Robinson *et al.*, 1977; Brunner *et al.*, 1983).

Low level commensal carriage of *U. urealyticum* in asymptomatic individuals has been reported which is both age and sex-dependent (males aged 50 or less, 21%; aged over 50, 7%; females aged 50 or less, 39%; aged over 50, 16%) (Taylor-Robinson, personal communication, 1988), but the titres of organisms recovered have been relatively low.

Ureaplasmas have been isolated with statistical significance more frequently from patients with N.G.U. compared to control patients in about one half of all studies reported and the selection of inappropriate controls has probably contributed most to the differences seen between one study and another. It is desirable that patients in the control group are free from N.G.U. and have sexual habits similar to those of patients in the study group, since sexual experience appears to be a major determinant of colonisation with ureaplasmas (McCormack *et al.*, 1973). It is also desirable that other pathogens such as *C. trachomatis* are tested for.

Wong *et al.* (1977) reported that there was an association between *U. urealyticum* only when *C. trachomatis*-positive patients were excluded from the study, a finding corroborated by Bowie *et al.* (1977). Further supporting evidence that chlamydia infection is inhibitory to the participation of ureaplasmas in the disease process has come from the work of Holmes *et al.* (1975) and of Paavonen *et al.* (1978).

In 1979, Taylor-Robinson *et al.* isolated chlamydia from 36% of ureaplasma-positive patients and found a similar isolation rate in ureaplasma-negative patients. Conversely, they isolated ureaplasmas from 52.5% chlamydia-positive patients and from 53% of chlamydia-negative patients so disputing these earlier claims.

The low level commensal carriage of *U. urealyticum* in the urethra of asymptomatic males may suggest that the disease process could depend upon an

explosion in the number of organisms present and also raises the possibility that some serotypes may be commensal whilst others are predominately pathogenic. In support of this latter theory, Shepard (1974) isolated serotype 4 twice as frequently from men with N.G.U. as from those who were asymptomatic. However, Hewish (1986) studied various isolates by immunofluoresence and concluded that colonisation above the urethra and association with urinary tract disease appeared to be serotype-independent.

Predisposing factors, such as lack of mucosal immunity, may exist in those individuals who do develop disease since a ureaplasma infection of the urethra of a 23 year old hypogammaglobulinaemic male patient was reported (Webster *et al.*, 1982). The patient developed a chronic urethral discharge from which greater than or equal to 5×10^8 c.c.u. ml⁻¹ ureaplasmas, but no chlamydia were isolated. Ureaplasmas may produce urethritis which resolves spontaneously while the organisms still persist or disease may only develop on initial exposure to the organism. If so, studies of individuals attending sexually-transmitted disease clinics are not likely to provide useful information and unfortunately, almost all studies to date have focused on these patients.

With respect to the number of organisms involved, one of the failures of previous studies is that they have been qualitative rather than quantitative.

There are two early reports by Bowie *et al.* (1977) and by Weidner *et al.* (1978) which have quantitative data supporting this theory that large numbers of organisms are involved. In such studies, it is necessary to be aware of the possibility that more organisms may be isolated from a patient who has a discharge than from one who has not.

Whereas the significance of ureaplasmas in N.G.U. seems no longer to be disputed, the degree of their involvement is still unknown. However, perhaps the most compelling evidence that ureaplasmas are associated with N.G.U. came when two

workers inoculated themselves intraurethrally with 5×10^4 organisms of two different strains of serotype 5 which had been isolated from two patients with N.G.U. Both men developed urethritis, although one developed only a mild condition. Both men responded to treatment with minocycline after which the organisms could no longer be isolated from the meatus, urine or semen. In the mild case of N.G.U., urinary threads persisted for at least 6 months after treatment and it appeared that the ureaplasmas had infected the urinary tract (Taylor-Robertson et al., 1971).

Ureaplasma antibodies were detected by an ELISA technique which had broad serotype cross-reactivity; a significant change in antibody levels for one or more antibody classes was detected in 12/18 NGU patients and 10/12 of the individuals had a change in the Ig M class suggestive of an active infection (Brown *et al.*, 1983). In previous studies, the Metabolic Inhibition test was often used and because of its serotype specificity this procedure would not have been able to detect more than a small proportion of responses and this may account for the small numbers of patients having a detectable antibody response. In a recent study by Deodhar and Gogate (1988) *U. urealyticum* was isolated from 48 % of patients with N.G.U. and an antibody response in these patients was shown using an ELISA test. *C. trachomatis* was detected in 24 % of patients with this condition, confirming that both organisms play a role in N.G.U.

Another useful approach to understanding the role of ureaplasmas in this disease is to treat patients with antibiotics which have differential activity against *U. urealyticum* and *C. trachomatis*. When patients with N.G.U. were treated with rifampicin, which acts against chlamydiae but not ureaplasmas, ureaplasmas were isolated from 55 of 68 men whose tests for ureaplasmas had initially been positive. Of these men, 44% did not recover, whereas only 8% of men whose ureaplasmas disappeared were not cured (Coufalik, 1979).

1.6.2. INFERTILITY.

Adsorption of human spermatozoa to colonies of mycoplasmas was reported in 1967 (Taylor-Robinson and Manchee) but the significance of this was unknown. Ericsson and Baker (1967) isolated ureaplasmas in large numbers from the ejaculates and cervical secretions of a selected group of patients with unexplained infertility and these workers suggested that the ureaplasmas were attached to the sperm cells. Evidence to support this came from scanning electron microscopy (Gnarpe and Friberg, 1973) which revealed ureaplasma colonies apparently originating from the anterior or middle region of the spermatozoa. These workers had previously reported that pregnancies were achieved in 30 % of infertile couples after eradication of ureaplasmas with antibiotics, but unfortunately, no control group was studied. A study by Toth *et al.* (1983) also reported that treatment with doxycycline improved fertility due to the eradication of ureaplasmas. However, no attempts to isolate bacteria or chlamydiae were made, and as this drug will also eradicate these organisms, no conclusions can be drawn from this study.

Contrary to this, Harrison *et al.* (1975) reported that the rate of conception in 28 infertile couples of unknown cause, but who were contaminated with ureaplasmas and treated with doxycycline, was no higher than in similar couples treated with a placebo even though the drug eliminated ureaplasmas from 27 of the 28 infertile couples. Taylor-Robinson and McCormack (1980) reported similar ureaplasma isolation rates from the semen of males with normal and low fertility.

Semen specimens from 625 men with infertility of unknown aetiology were examined and of those with ureaplasma 'infections', low numbers, poor levels of motility and an increased number of aberrant forms were reported (Folwkes *et al.*, 1975a). These workers again suggested this was due to the attachment of ureaplasmas to the spermatozoa which impedes normal motility (Folwkes *et al.*, 1975b). This was supported by O'Leary and Frick (1975) but they suggested the sperm

may also be inhibited in a biochemical sense as well as by physical hindrance. As ureaplasmas are reported as being present in a ratio of 1 organism to 10 spermatozoa, even in a sample of 'infertile semen', then it seems unlikely that these organisms could affect motility (Taylor-Robinson, 1986b). Since the organisms probably gain access to semen from the prostate, urethra or prepuce at the time of ejaculation, it would be surprising if the organisms could affect spermatogenesis or motility (Taylor-Robinson, 1986b).

To elucidate the mechanisms whereby ureaplasmas can interfere with the fertilisation process, a human sperm-hamster egg fertilisation test was studied (Busolo and Zanchetta, 1984; 1985) where human spermatozoa can fuse with zona-free hamster eggs which is considered a measure of spermatozoa fertilising ability. No significant change in morphology or motility of the sperm was seen after overnight incubation in the presence of ureaplasmas (serotypes 1-8); However, the number of eggs penetrated was greatly reduced particularly after incubation with serotype 4 (6.3% compared to 55.6 % penetration rate seen with the control). Serotypes 6 and 8 also caused reduced penetration rates and these three serotypes correspond to the serotypes involved in spontaneous abortion reported by Quinn *et al.* (1983) (section 1.6.4.2).

Ureaplasmas are known to produce ammonia during active hydrolysis of urea and this has been shown to be toxic for cells. Busolo and Zanchetta (1985) reported no such effect on either hamster eggs or spermatozoa although it was unclear as to whether urea was present in the test. Thus, they suggested that neither a cytotoxic effect of ureaplasmas on gametes or a masking of the binding sites on the egg surface were due to the decrease in penetration. They suggested infertility could be due to the effect of ureaplasma in the vagina influencing the maturation of the spermatozoa by the production of unknown factor(s).

Urogenital tract infections have also been associated with the production of sperm

antibodies and these in turn have been associated with a reduction in male fertility (Whitkin and Toth, 1983). Whether ureaplasmas could stimulate sperm antibodies is unknown but it has been reported that no such correlation between the presence of ureaplasmas in semen and sperm antibodies exists. (Upadhyaya *et al.*, 1984).

Most of the above studies have been deficient since they have failed to exclude the involvement of other microorganisms, particularly chlamydiae; the antibiotic studies have not included placebo-treated controls and there has been a failure to match the control groups for factors known to influence ureaplasma colonisation.

If ureaplasmas play a role in prostatitis and if prostatitis is a cause of infertility, then by implication, ureaplasmas may be a cause of infertility.

Semen could become contaminated on passage through the urethra (Taylor-Robinson, 1986b), but the residence time is low. Spermatozoa may again come into contact with ureaplasmas in the lower female genital tract where residence time may permit a reduction in their mobility. In this connection, Stray-Pedersen *et al.* (1978) recovered ureaplasmas more often from endometrial aspirates from infertile than from fertile women.

Thus the role of ureaplasmas in infertility is unresolved and examination of female endometrial specimens may be more meaningful than examination of those from the lower genital tract. Although, Cassell *et al.* (1983) sought to recover *U. urealyticum* from the endometrium of infertile patients undergoing laparoscopy and from control patients, these workers attempted to prevent cervical contamination of the specimens and no significant difference in *U. urealyticum* recovery was found between the infertile and control patients.

Properly controlled treatment studies in which the role of other microorganisms are considered should be carried out but it must be recognised that eradication of infection may not necessarily reverse the damage that the organisms may have

caused.

1.6.3. ARTHRITIS.

Sexually acquired reactive arthritis (SARA) develops in a low percentage of males with N.G.U. where *U. urealyticum* has been isolated from the urogenital tract (Cole *et al.*, 1985) but the isolation of these organisms from diseased joints has met with little success. More emphasis should be placed on the detection of mycoplasma antigens as immune complexes may be involved in the development of arthritis.

U. urealyticum has been isolated from the joints of at least three hypogammaglobulinaemic patients who have had septic arthritis. The lack of antibody in hypogammaglobulinaemic patients probably results in the failure of the mycoplasmas to be 'neutralised' and accounts for the diminished ability of the patients to cope with these organisms escaping haematogenously from the genital tract. Furthermore, *U. urealyticum* and other mycoplasmas are ingested by neutrophils in the absence of opsonins, indicated by the fact that they are able to trigger the release of chemiluminescence from these cells; ureaplasmas are not killed during this process and it is possible that carriage occurs within phagocytes to various sites (Taylor-Robinson *et al.*, 1986a).

There is also some evidence that pregnancy may be accompanied by T-cell suppression, possibly allowing the development of opportunistic infections, which may explain why some mothers develop arthritis soon after childbirth. Thus, the role of ureaplasmas in arthritis is as yet unknown, but a study by Barile *et al.* (1988) isolated a strain of *U. urealyticum* from a joint in a patient with septic arthritis which caused a severe and protracted arthritis in chimpanzees. In contrast, a strain isolated from a patient with Reiter's disease, after frequent passage, failed to produce disease in chimpanzees.

1.6.4. PREGNANCY DISORDERS.

Commensal carriage of *U. urealyticum* in sexually mature, asymptomatic women has been mentioned previously (see section 1.6.1.). In the female, colonisation is thought to be predominantly linked with younger age groups (16-50), lower socio-economic status, sexual activity particularly with multiple partners, black ethnicity and oral contraceptives (McCormack, 1983). It has been suggested (Furr and Taylor-Robinson, 1988) that ureaplasmas are not found at a constant rate during the menstrual cycle suggesting that hormones may influence their colonisation or recovery. Female mice pre-treated with oestrogen are more susceptible to genital tract infection with *U. urealyticum* compared to untreated mice. Pregnant women also appear to be colonised more frequently with ureaplasma compared to non-pregnant women (33% compared with 22.7%) (Furr and Taylor-Robinson, 1988).

U. urealyticum may be transmitted to 38% of babies born to mothers whose genital tract is infected with this organism, though colonisation of the neonate appears to be transient with a sharp decline in isolation rates after 3 months of age (McCormack and Taylor-Robinson, 1984). *U. urealyticum* has been associated with a number of disorders of pregnancy:-

1.6.4.1. CHORIOAMNIONITIS.

The chorioamnion denotes the space lying between the amnion (the foetal surface of the sac) and the chorion (the maternal surface), and chorioamnionitis is an infection of these membranes. In chorioamnionitis, polymorphonuclear leucocytes are seen in various degrees of abundance lying within this space; they seldom extend through the foetal surface of the amnion but they may go deep into the chorion, even to the intervillous space of the placenta. This condition has been associated with premature rupture of membranes (PROM) (Gravett and Eschenbach, 1986), premature delivery, foetal morbidity and mortality, eg neonatal pneumonia (see later) (Kundsin et.al 1984;

Driscoll, 1986).

U. urealyticum has been implicated as one of the causes of this condition. In two studies, (Kundsin *et al.*, 1984; Quinn *et al.*, 1985) workers rigorously sought the presence of other fastidious microorganisms but *U. urealyticum* alone was significantly associated with the presence of inflammation. The placental studies took into account the time of membrane rupture but they did not consider the duration of labour nor the presence or absence of *U. urealyticum* in the cervix or vagina of study and control patients. This is important as recent evidence suggests that bacteria from the cervix or vagina often invade the amniotic fluid at the onset of labour and prior to membrane rupture (Cassell *et al.*, 1986).

A more recent study has also associated *U. urealyticum* with chorioamnionitis (Quinn *et al.*, 1987). It appears that the organism does not directly cause the associated PROM or prematurity and they suggest that this is due to the leucocytic response as neutrophils secrete the prostoglandin PGF₂, a potent promoter of uterine contraction (Charles and Hurry, 1983).

More recently, Quinn (1988) observed chorioamnionitis in 82.6 % of women whose babies were born dead or died soon after birth compared to only 5 % of babies from women not suffering from this condition. *U. urealyticum* was isolated from the placenta in 56.5 % of these cases compared to 0 % of the controls.

Chorioamnionitis is a condition that cannot be diagnosed accurately until after the event and as a consequence, it may be important to recognise those women at risk and treat them accordingly. It has been suggested that ureaplasmas are more likely to cause complications where the women are undernourished or when the membranes rupture prematurely (Naeye and Blanc, 1974).

1.6.4.2. SPONTANEOUS ABORTION AND STILLBIRTH.

Kundsin *et al.* (1967) isolated *U. urealyticum* from the chorion, amnion and decidua of a spontaneously aborted foetus where the foetal membranes and umbilical cord were also severely inflamed and no other organism was isolated. *U. urealyticum* was not isolated from the lungs or liver of the foetus but this organism was isolated from a cervical swab and from urine sediments of both the husband and wife. This suggested that the aborted foetus could have become contaminated during birth.

The ureaplasmas isolated from aborted fetuses and stillbirths cannot be attributed solely to superficial contamination because the organisms have been isolated from the lung, brain, heart and viscera (Taylor-Robinson and McCormack, 1979).

Nevertheless, many couples carrying *U. urealyticum* have normal pregnancies. This may be related to a difference in the pathogenicity of the 14 serotypes since Naessens (1988) suggested that *U. urealyticum* serotype 4 was isolated more frequently from patients with a history of recurrent miscarriages compared to normal pregnant women, or some other predisposing factor may be involved.

Samples from cervical mucus and endometrial tissue were examined for the presence of *U. urealyticum* in patients with a history of habitual abortion and in a suitable control group (Stray-Pederson *et al.*, 1978). These workers found colonisation of *U. urealyticum* in the cervix was common in both groups, whereas colonisation of the endometrium was found to be significantly more frequent among the habitual aborters (28%) than among the control subjects (7%). Treatment with doxycycline eradicated the ureaplasmas from both the cervix and the endometrium which apparently led to an improvement in the outcome of pregnancy. Since doxycycline is a broad spectrum antibiotic, this observation does not permit any definite conclusions as to the role of ureaplasmas in spontaneous abortion.

Quinn *et al.* (1983) also reported high colonisation rates of *U. urealyticum* in

couples with histories of pregnancy wastage compared to control groups. Serological studies revealed that at delivery, 42.9 % of infants of mothers with history of pregnancy loss had fourfold elevations in titers to ureaplasmas (particularly to serotypes 6 & 8) above the mother's level. Some mothers with pregnancy wastage history also had elevated titers (particularly serotypes 4 & 8) above the infants, thus both mothers and fetuses responded immunologically to the presence of *U. urealyticum*, suggesting the presence of an ongoing infection.

Furthermore, antibiotic therapy has played some part in elucidating the role of *U. urealyticum* with these conditions. Kunsdin (1970), has described successful pregnancies after antibiotic treatment in women who were colonised with ureaplasmas and who had frequent spontaneous abortions, but again, other bacteria may have been eliminated.

The mechanism(s) for the association of spontaneous abortion and ureaplasma infection is/are unknown. The high frequency of abnormal chromosome patterns seen in aborted foetal tissue may reflect ureaplasma infection which in experimental studies has been noted to be able to induce chromosomal aberrations such as chromatid gaps, breaks and tetraploidy (Kundsine *et al.*, 1971).

Harrison *et al.* (1986) studied two groups of pregnant women and isolated *U. urealyticum* from cervical samples from 72.5 % and 81.2 % respectively. The overall incidence of spontaneous abortion in these groups were 9.7 % and 5.5 % and the incidence of spontaneous abortion in *U. urealyticum*-positive women was 9.4 % and 5.6 %, neither of which was statistically important. This suggested that ureaplasma colonisation is not an important factor in spontaneous abortion. However, if the results reported by Stray-Pederson *et al.* (1978) are correct, these workers may have seen more meaningful results if cultures had been taken from the endometrium.

A more recent study by Robertson *et al.* (1986) isolated *U. urealyticum* from 24 % of spontaneous abortions obtained by unaided vaginal delivery and from only 8% of

therapeutic abortions by suction curettage suggesting that ureaplasmas do cause spontaneous abortions, at least in some individuals. The isolates were serotyped using the modified indirect colony epifluorescent test but no serotype(s) appeared to be predominant perhaps suggesting that differences lie in the host rather than with the organism.

In 1980, Embree *et al.* cultured placentas for *U. urealyticum* from a high risk pregnancy group and a control group. Ureaplasmas were recovered more frequently from the high-risk-group compared to the control group and isolation was associated with polymorphonuclear leukocyte infiltration of placental membranes, foetal surfaces and umbilical cords. The contaminated placentas were frequently from cases of spontaneous abortion, stillbirths, early neonatal deaths as well as from infants of lowbirth weight and mothers with premature rupture of membranes. *U. urealyticum* was also isolated from placentas in which the membranes had first been ruptured at delivery by caesarean section suggesting that intact membranes do not provide an impenetrable barrier to infections of the placenta or of the foetus.

Ureaplasmas were isolated from the placenta or lung in 78.8 % of perinatal death cases compared to 32.3 % of controls. Elevated antibody response to *U. urealyticum* was detected in 45.8 % of these foetuses compared to 0 % of controls, suggesting that these organisms do cause infection 'in utero' leading to death (Quinn *et al.*, 1985).

In 1986 , Quinn studied a further 22 cases of stillbirth and detected an antibody response of greater than or equal to 1/32 to at least one of eight serotypes in 77.3 % of cases.

Placenta, liver and lung tissue were cultured for ureaplasmas and other microorganisms from 432 stillborn and neonatal autopsies. Ureaplasmas were isolated from 8.3 % of cases- 2.2 % of placental specimens, 7.4 % of lung specimens and 0.8% of liver specimens (Madan *et al.*, 1988).

The above studies suggest that ureaplasmas can and do cause spontaneous abortions and stillbirths in a small percentage of women, but exactly how this occurs is as yet unknown. In this respect, phospholipase activity has been found in *U. urealyticum* serotypes 3,4 and 8 (Desilva and Quinn, 1986) and it has been suggested that these enzymes play a role in this condition (see section 1.7.3).

1.6.4.3. LOW BIRTH WEIGHT.

Premature birth is the most common cause of infant death and premature labour remains unexplained in many cases.

Approximately 15% of randomly selected new born infants were found to have nasal or pharyngeal colonisation, mainly with ureaplasmas, and such colonisation correlated statistically with lower mean birth weights (2605g) (Klein *et al.*, 1969). However, these workers suggested this colonisation occurred during the birth process and was not the cause of the low birth weight.

Braun *et al.* (1971) also recovered ureaplasmas more frequently from women who gave birth to infants of low weight compared to mothers who gave birth to average size babies. In this study, 79 % of women were contaminated with *U. urealyticum* and since only 8.5 % of all babies weighed less than 2500g, it is apparent that other factors are of importance in influencing birth weight.

Kundsinn *et al.* (1984) also associated *U. urealyticum* with infants weighing less than 2500g and also with birth before 36 weeks gestation.

In 1986, Kass *et al.* reported that women who experienced a 4-fold or greater rise in antibody titre to any single strain of *U. urealyticum*, had a low birth weight rate of 30 %, whereas women who did not experience a rise in antibody titre had a low birth weight rate of 7.3 %. Where there was a significant rise in antibody titre, the titre rise was specific for a serologic type other than those for which the women had

pre-existing antibody. This finding suggests that immunity to *U. urealyticum* is type-specific and that low birth weight is associated with antibody rises when they occur in relation to invasion with a serovar that has not previously elicited antibody formation in the pregnant women.

Furthermore, preterm delivery of low birth weight infants seems to be a pre-disposing factor for a number of other conditions:-

(i) Neonatal pneumonia: (Reviewed by Rudd *et al.*, 1986; Cassell *et al.*, 1988).

Respiratory disease is the most common cause of perinatal morbidity and mortality, approximately 20 % of stillborn babies and infants dying within 72 hours of age have histological evidence of pneumonia. There is some evidence to suggest that congenital pneumonia, or pneumonia acquired during birth, is almost always accompanied by chorioamnionitis, (see section 1.6.4.1), (Naeye *et al.*, 1971; Driscoll, 1986).

The diagnosis of lower respiratory tract disease in newborn babies is difficult as cultures from the lung are not easily obtained, whereas cultures from the throat, nasopharynx and blood are unrevealing or misleading.

In 1971, Romano *et al.* reported the isolation of *U. urealyticum* from the placenta and from the heart and lungs of a foetus with intrauterine bronchopneumonia in the fifth month of pregnancy. Although there was no definite evidence that the ureaplasma isolated from the lung of the foetus was involved in the pathogenesis of the pneumonia, cultures for bacteria, chlamydiae and viruses were negative. An increase in antibody against *U. urealyticum* was also seen in the post-partum serum obtained from the mother 4 weeks after delivery.

Twenty three cases of congenital pneumonia in Ethiopian infants, who later died, were associated with *U. urealyticum* and again no other organisms were isolated and no other recognised disorder was diagnosed. Although these infants were born near

term they were undergrown for gestational age, presumably due to maternal undernutrition (Tafari *et al.*, 1976).

Stagno (1981) also isolated *U. urealyticum* from 21 % of infants (2-12 wks) suffering from pneumonia although in most cases, other pathogens such as *Pneumocystis carinii*, cytomegalovirus, *Chlamydia trachomatis* and *Bordetella pertussis* were co-isolated. This suggested that *U. urealyticum* may be a secondary and not a primary pathogen of the respiratory tract in this age group. Taylor-Robinson *et al.* (1984) isolated *U. urealyticum* from 26 % of babies without respiratory disease but with other complications and from 22.5 % of babies with respiratory disease. The organisms were also isolated from 19 % of healthy babies. All the above infants were delivered vaginally and could have become contaminated in this way. Sixteen of these babies were delivered by caesarean section, only one of which was contaminated with *U. urealyticum*, whereas seven babies developed respiratory disease. Thus, these authors argued that transient colonisation occurs in healthy infants whereas a more sustained infection may occur in a few babies with respiratory disease.

As up to 80 % of pregnant women and 30 % of neonates may be colonised with ureaplasmas, Quinn *et al.* (1983) studied 'true infection' by measuring antibody response to 8 serotypes using the Metabolic Inhibition test in both mother and infant in 21 cases of neonatal respiratory disease and 24 normal cases. They suggested that a 4-fold difference in antibody titer between mother and infant before 20 days of age, was evidence of a serologic response of the infant to *U. urealyticum*. This response was seen in 11/21 infants with respiratory disease and in none of the 24 normal term babies. Antibody to serotypes 4,7 and 8 predominated suggesting these serotypes may be more important in terms of this condition.

There was no definite evidence that these babies were infected or colonised with *U. urealyticum* as isolation of the organism was not attempted and it has not yet been established whether mere colonisation with *U. urealyticum* produces an antibody

response (Rudd *et al.*, 1986).

There was also no definition or documentation of respiratory disease. Infants with respiratory disease often receive almost daily blood transfusions and as a result serology must be interpreted with caution, especially in the absence of culture data (Cassell *et al.*, 1988).

In a later study, Quinn *et al.* (1985) reported a case of fatal neonatal pneumonia. The lungs from the infant were inflamed and immunofluorescent staining of the lung tissue was positive with antisera to *U. urealyticum*, serotype 8, correlating with the serotype isolated from the lung where no other microorganisms were isolated. Antisera to serotypes 1 and 7 were also used but gave negative staining. The trachea from the infant was devoid of cilia in places or when present, were short and stubby which may be associated with *U. urealyticum* infection as observed in previously aborted fetuses (Dische *et al.*, 1979). The infant's antibody titre to *U. urealyticum*, serotype 8 was 1:64 at 24 hours of age and 1:128 at autopsy while the mother's was only 1:16. Infection of the foetus may have occurred because the mother did not have sufficient specific Ig G antibody to supply transplacental Ig G for the protection of the neonate.

A strong association between chorioamnionitis caused by any agent and congenital pneumonia, prematurity and perinatal morbidity and mortality has been reported (Naeye, 1975). *U. urealyticum* has been linked with chorioamnionitis and individual case reports suggest that in some patients it is causal with respect to chorioamnionitis and also congenital pneumonia (Cassell *et al.*, 1983).

More recently (Cassell *et al.*, 1988), *U. urealyticum* was isolated most commonly from endotracheal aspirates from low birth weight infants (2500g or less) with evidence of respiratory disease. 14 % of isolates were from infants born by caesarean section with intact membranes, thus indicating the infection had occurred 'in utero'. These organisms were recovered in pure culture in numbers greater than 10^3 c.f.u.

ml⁻¹ from 85% of the infants and the organism was also isolated by blood culture from 26% of these infants. These workers suggested that those infants weighing less than 1000g with *U. urealyticum* infection of the lower respiratory tract were twice more likely to have chronic lung disease or to die than were infants with similar birthweight but who were uninfected, or infants weighing more than 1000g.

Sanchez and Regan (1988) reported that respiratory distress in low birth weight infants was not associated with *U. urealyticum* colonisation. However, of the infants colonised with *U. urealyticum*, 30 % developed chronic lung disease whereas only 8 % of those not colonised developed chronic lung disease.

U. urealyticum, serotype 10, which was isolated in the above study (Cassell *et al.*, 1988), was used to inoculate newborn and 14 day-old mice (Rudd *et al.*, 1989). Serotype 1 was also studied and this serotype was isolated in 1983 (Cassell *et al.*) from amniotic fluid and from the lungs of a newborn infant suffering from pneumonia. These organisms were shown to produce an acute, self-limiting interstitial pneumonia in newborn mice, with organisms localised within the alveoli in areas of inflammation. It was discovered that 14 day-old mice were less susceptible to either colonisation or disease.

These results are compatible with the apparent age-related susceptibility observed in the recent studies discussed previously. The reasons for this are not known but they may be linked with the development of the hosts defences. In mice, ureaplasma were found within macrophages by 8-12 days of age, and as 14 day-old mice did not develop alveolar lesions, it was suggested that differences in macrophage function may be important in causing susceptibility to *U. urealyticum* pneumonia. However, 14 day-old mice were also more resistant to colonisation of the upper respiratory tract, suggesting other factors may also be involved in the age-related susceptibility. It has also been reported that mice infected with *U. urealyticum* and treated with oxygen are more susceptible to a prolonged pneumonia or death compared to mice not given

oxygen (Crouse *et al.*, 1988), and these workers suggested the same may be true for human neonates.

It is not clear whether the mouse model has significant relevance to newborn human infants. Further characterisation of the mouse model of *U. urealyticum* should help define both host and organism factors that are involved in disease susceptibility and disease pathogenicity.

Thus, it would appear that ureaplasmas do play a role in respiratory disease, especially in preterm infants, but to what extent and how, is still unknown.

(ii) Infection of the central nervous system (CNS) in preterm infants:

Waites *et al.* (1988) isolated *U. urealyticum* from 6 preterm infants with severe intraventricular haemorrhage and from 3 with hydrocephalus and in 4 of these babies, multiple isolations were made over several weeks. The authors have suggested that *U. urealyticum* is one of the most common organisms isolated from the cerebro spinal fluid (CSF) of newborn infants with low birth weights.

In a more recent study, *U. urealyticum* alone was isolated from 8 % of CSF's from preterm infants and was associated with severe intraventricular haemorrhage and hydrocephalus (Waites *et al.*, 1988).

Until more studies have been done, the role of these organisms in infections of the CNS must be treated with caution.

1.6.4.4. PELVIC INFLAMMATORY DISEASE (PID).

Chlamydia trachomatis, *Neisseria gonorrhoeae* and anaerobic bacteria are among the most causative agents of PID. Ureaplasmas have also been considered an aetiological agent of PID, and although the proportion of such cases is unclear, ureaplasmas are often co-isolated with these organisms (Reviewed by Sweet, 1986).

The reported cervical isolation rates for *U. urealyticum* range from 19 to 81 %. Both Mardh and Westrom (1970) and Eschenbach *et al.* (1975) noted that the cervical isolation rates from PID patients and sexually active controls were similar.

In a group of 88 patients with acute PID, *M. hominis* was recovered from the endometrium in 34 patients (39 %) and *U. urealyticum* was recovered in only 13 patients (15 %) (Sweet, 1986).

In an attempt to demonstrate an etiologic role for genital mycoplasma in acute PID, serological studies of ureaplasma antibodies have been undertaken, and Eschenbach reported a 4-fold rise in antibody titre to *U. urealyticum* in 17 % of patients with acute PID.

Sweet *et al.* (1981) noted that *U. urealyticum* was recovered from the fallopian tubes of patients suffering from an initial episode of PID as well as from those having recurrent episodes. Isolation of *U. urealyticum* from the fallopian tube occurred only from patients who had been symptomatic for greater than 72 hours and this data may suggest that isolation of *U. urealyticum* from fallopian tubes may reflect secondary invasion or colonisation after damage has occurred to the fallopian tube epithelium following infection with *N. gonorrhoeae*, *C. trachomatis* or anaerobic bacteria.

1.6.4.5. POSTPARTUM FEVER.

Postpartum fever can be classified into that which occurs early (less than 48 hrs postpartum) and that which occurs late (3 days to 6 weeks postpartum). Causes of early postpartum fever include endometritis (usually following a caesarean section), urinary tract disease, a rapidly developing wound infection, etc. Frequent causes of late postpartum include endometritis, abscess formation and urinary infections.

U. urealyticum and other organisms colonise placental membranes and amniotic fluid (section 1.6.4(a)) either spontaneously or following rupture of the membrane and

organisms present in these sites can reach the placental bed where they may enter the blood stream. *U. urealyticum* was first associated with postpartum fever in 1971 (Sompolinsky *et al.*)

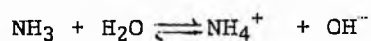
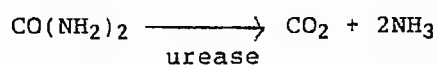
Genital mycoplasmas have been recovered from the blood in 2/13 (15 %) women within 2 minutes of delivery, in 23/272 (8%) women within 2 to 10 minutes after delivery and in 1/42 (2%) of women 11 to 45 minutes after delivery (McCormack *et al.*, 1975). Nevertheless, only 40 % of these women with this transient invasion of *U. urealyticum* developed postpartum fever.

Eschenbach (1986) suggests that approximately 20 % of women with early postpartum endometritis have evidence of endometrial *U. urealyticum* infection and about 5 % of the women have *U. urealyticum* recovered from the blood. Thus genital mycoplasmas appear to cause a small but definite number of cases of postpartum fever.

1.6.5. UPPER URINARY TRACT DISEASE.

1.6.5.1. URINARY TRACT STONES.

Urinary tract stones composed of struvite and carbonate-apatite (see below) account for 20% of all urinary tract stones and are often known as infectious urinary tract stones. These stones appear to be induced by the enzymatic breakdown of urea by bacterial ureases which split urea into ammonia and carbon dioxide (CO₂) (Grenabo *et al.* 1984; Griffith *et al.*, 1976). Non infectious stones are composed of oxalate, a mixture of oxalate and phosphate or uric acid.



This enzymic reaction is important in the urinary tract due to the high substrate level of urea (25–30 mg ml⁻¹) in urine. Hydrolysis of urea leads to hyperammoniurea and the alkalisation of urine which causes hypersaturation with respect to struvite and calcium phosphate, and this leads to crystallisation of struvite and apatite (Takebe *et al.*, 1984).

Calcium and Magnesium Phosphates:-

Struvite	(MgNH ₄ PO ₄ ·6H ₂ O)
Carbonate-apatite	(Ca ₁₀ (PO ₄) ₆ CO ₃)
Brushite	(CaHPO ₄)
Hydroxyapatite	(Ca ₁₀ (PO ₄) ₆ (OH) ₂)

Proteus species and to a lesser extent *Klebsiellas*, *Pseudomonads* and *Staphylococcus* species, are the usual cause of stones in urine but *U. urealyticum* has also been shown to induce crystallisation of struvite and other calcium phosphates *in vitro* in artificial urine (Grenabo *et al.*, 1984). In this study, *U. urealyticum* (10⁴ organisms ml⁻¹) were added to synthetic urine, pH 5.7, and after 10 hours the pH had risen to 8.1. A clearly visible encrustation of brushite and struvite (23 µg) was seen although no more than 10⁵ organisms ml⁻¹ were ever isolated. In comparison, 10⁴ organisms ml⁻¹ of *Proteus mirabilis* were added to synthetic urine as above, and within 3 hours, the pH had risen to 9.0 with 10⁸ organisms ml⁻¹ isolated, and after 10 hours, a pH of 9.0 was still seen with 2082 µg of struvite formed.

Although, *P. mirabilis* caused a more pronounced crystallisation than *U. urealyticum* this could have been due to a difference in growth rate. *P. mirabilis* grows optimally at pH 7.0–9.0 which is within the pH of the urine, whereas *U. urealyticum* grows optimally at pH 6.0, and as the pH of the urine rose to 8.1, this would greatly reduce the rate of growth. It should be noted that no crystallisation was seen with either organism when the urease inhibitor acetohydroxamic acid (AHA) was added.

This study suggests that *U. urealyticum* can initiate the formation of infectious stones and the above was confirmed by Takebe *et al.* (1984), where 10^8 cells ml^{-1} were inoculated into normal human urine and a white precipitate of iokite and struvite was formed. Again stone formation was prevented with the addition of urease inhibitors.

Urinary calculi have a strong tendency to recur despite surgery and antibiotic treatment and this could be due to the presence of *U. urealyticum* as this organism is now resistant to many of the antibiotics used for treating infections of the upper urinary tract (Pettersen, 1983; Grenabo *et al.*, 1984).

Renal calculi, composed of struvite have also been induced experimentally in 78 % of rats after direct inoculation of *U. urealyticum*, serotype 8, ($10^5 - 10^6$ ccu ml^{-1}) into the bladder and renal medulla (Friedlander and Braude, 1974). These workers indicated that viable ureaplasmas were not required for stone formation. In contrast, heat-killed *P. mirabillis* did not produce bladder stones in the rats and these workers suggested that stones are formed due to the ability of the dead ureaplasmas to act as a nidus, as they adsorb urinary mucoprotein which is the principle component of the organic matrix of stones.

U. urealyticum which is an organism not normally isolated from the upper urinary tract, has also been isolated from infectious stones recovered by surgery from 6 of 15 patients (Pettersen, 1983). In 4 of these patients, no other urease-producing organisms were isolated from either the stone or the urine sampled from the renal pelvis.

The major question remaining concerning the role of this organism in the production of urinary calculi is the frequency with which the organism reaches the kidney, the predisposing factors that allow this to occur, and the relative frequency of renal calculi induced by this organism compared to those induced by other organisms.

1.6.6. REFLUX NEPHROPATHY (CHRONIC ATROPHIC PYELONEPHRITIS).

U. urealyticum has been infrequently isolated from the bladder and upper urinary tract, sites from which genital and urethral contamination can be rigorously excluded (Birch *et al.*, 1981). However, clinical studies suggest that *U. urealyticum* may be an upper urinary tract pathogen in some patients with reflux nephropathy and impaired renal disease (For a review see Krieger and Kenny, 1986). This may suggest that ureaplasma colonisation contributes to progressive renal disease and again it may be possible that only a few serotypes are involved.

Hewish *et al.* (1986) serotyped all isolates of *U. urealyticum* isolated from the bladders and upper urinary tract of 124 patients in order to determine whether particular serotypes were associated with infection at different levels within the urinary tract, or with a particular urinary tract disease with emphasis on reflux nephropathy. Serotype 4 was recovered most frequently (35 %) whereas serotypes 5 and 8 were infrequently isolated. These workers concluded that colonisation above the urethra and association with urinary tract disease appeared to be serotype-independent.

Unfortunately, isolation of ureaplasmas from patients with upper urinary tract disease does not prove that ureaplasmas are the causative agents as other organisms are recovered in many cases.

The use of animal models enables workers to study the effects of pure culture under controlled conditions and obtaining suitable control groups is straight-forward compared to clinical studies.

Using an obstructed canine upper urinary tract (Krieger and Kenny, 1986), the inoculation of *U. urealyticum*, serotype 8, caused interstitial inflammation (pyelonephritis) and a rise in anti-ureaplasma antibody was noted.

Using a rat kidney model (Pickering and Birch, 1989), *U. urealyticum*, serotype 8 was found to be capable of persisting in the rat kidney and was recoverable from the infected kidney for up to 24 weeks after inoculation of 10^7 organisms. Animals with persistent infections exhibited a humoral response involving Ig M and Ig G antibodies. These workers suggest that *U. urealyticum* is capable in giving rise to chronic infection in the kidneys of rats following challenge via the ascending route. This is in agreement with the findings in patients who have been shown to yield the same ureaplasma serotype from the bladder for more than 12 months (Hewish *et al.*, 1986).

How useful these 'artificial' animal models are in terms of human disease is unclear as interspecies barriers may exist for colonisation or production of disease in other animals by human strains. These studies suggest that infections only occur when high numbers of organisms are injected and whether these numbers are reached in humans, and if so, how often this occurs, is as yet unknown.

1.7. VIRULENCE MECHANISMS.

There is now a growing interest in *U. urealyticum* as an agent of human diseases, but little attention has been given to those characteristics of the organism which may contribute to the disease process(es). However, possible virulence factors were documented by Robertson (1986). Perhaps of most significance are the following:-

1.7.1. IMMUNOGLOBULIN A PROTEASE.

Immunoglobulin A (IgA) protease activity has been detected in only a limited number of the many pathogenic bacterial genera; e.g. *Streptococcus*, *Neisseria*, and *Haemophilus*. Furthermore this Ig A protease activity was associated only with the pathogenic species of these genera e.g. *N. meningitidis*, *N. gonorrhoeae* and *H. influenzae*, but not with the commensal species. However inconsistencies exist e.g. not all strains of *S. sanguis* have Ig A protease activity (Genco *et al.*, 1973).

Furthermore, although most strains of *H. influenzae* show activity, so do certain strains of *H. parahaemolyticus* and *H. parainfluenzae* which may have pathogenic potential but are species usually considered saprophytic. Thus the role of Ig A protease activity has yet to be resolved.

Secretory Ig A antibodies (sIg A) are considered a major defense mechanism at mucosal surfaces. Ig A protease cleaves Ig A at the hinge region into two fragments, Fab and Fc, and is considered to be a potential virulence factor for those organisms which gain entry at mucous membranes.

Robertson *et al.* (1984) isolated Ig A protease activity from all 14 serotypes and from 34 of 35 wild-type strains of *U. urealyticum* and this activity persisted in the presence of 25 mM EDTA but was sensitive to trypsin. Kapatais-Zoumbis *et al.* (1985) confirmed the presence of Ig A protease activity in *U. urealyticum* and showed this enzyme was specific for Ig A1 and not Ig A2.

Kilan and Freundt (1984) examined 52 strains representing the genera Mycoplasma, Ureaplasma, Acholeplasma and Spiroplasma for the ability to cause specific cleavage of human Ig A1. This activity was exclusively associated with strains of *U. urealyticum* although one of the three strains of *U. diversum*, the type strain (A417), degraded human Ig A1 extensively leaving no fragments that could be demonstrated by immunoelectrophoresis. This strain of *U. diversum* also extensively degraded bovine sIg A.

Also in 1984, Stemke *et al.* reported that 13 strains of ureaplasmas from seven animal species were unable to degrade human Ig A but unfortunately, *U. diversum* strains were not studied in terms of Ig A protease activity.

It has since been reported that Ig A protease from human *U. urealyticum* isolates is host Ig A-specific, similarly, Ig A protease from canine ureaplasmas will only cleave canine Ig A (Kapatais-Zoumbis *et al.*, 1985).

Establishment of the importance of Ig A protease activity in ureaplasma pathogenesis awaits further information on the distribution of specific activity and the elucidation of the role of Ig A at mucosal surfaces.

1.7.2. UREASE.

This enzyme has already been discussed with respect to urinary stone formation.

1.7.3. PHOSPHOLIPASES.

As discussed in the previous section, *U. urealyticum* has been associated with spontaneous abortion, stillbirth and prematurity.

A possible mechanism in the initiation of premature labour may be the effect of microbial phospholipase hydrolysis of placental membrane phospholipids to produce an increase in the amount of free arachidonic acid and consequently, an increase in the synthesis of prostaglandins (Quinn, personal communication, 1988).

Microbial phospholipases are known to have action on biomembranes and are capable of causing haemolysis, lethal toxicity and skin lesions.

It is possible that phospholipases A1/A2, producing large amounts of lysophospholipid quickly, may also play a role as cytotoxic agents in infectious processes.

DeSilva and Quinn (1986) assayed serotypes 3, 4 and 8 of *U. urealyticum* for the presence of phospholipase A1, A2 and C activities. Phospholipase A2 activity was 100-fold higher than that of phospholipase A1 and considerably higher in serotype 8 than in serotypes 3 and 4. However, the phospholipase A1 activities of serotypes 3 and 4 were comparable to the activities of other major perinatal pathogens, whereas the activity of serotype 8 was nearly three fold higher.

Phospholipase C activity has been detected in many microorganisms, such as *Bacillus cereus*, *Clostridium perfringens* and *Staphylococcus aureus* (Dennis, 1983), in serotypes 3,4 and 8 of *U. urealyticum* (DeSilva and Quinn, 1986) and in *Vibrio parahaemolyticus* (Guhathakurta *et al.*, 1988).

Phospholipase C has been purified from *Clostridium perfringens* and was reported to produce lesions in guinea pig skin (Guhathakurta *et al.*, 1988).

1.7.4. CELLULAR COMPOSITION.

Other features, such as the absence of a cell wall, may provide the opportunity for host cell surface components to bind directly to the ureaplasma membrane, thus shielding its antigenic determinants (Robertson, 1986).

Lipoglycans have been found in some mycoplasmas and are reported as being immunogenic, eliciting the production of the immunoglobulin M class of antibodies (Lynn *et al.*, 1980). Antigenic specificity is apparently defined by a repeating sequence of three sugar residues along the chains (Al-Samarrai and Smith, 1983). The lipoglycans also appear to be capable in modulating the immune response to both soluble and particulate antigens (Smith, 1984). Lipoglycans have been detected in serotypes 3,4 and 8 of *U. urealyticum*, all of which contained neutral sugars, fatty acids, glycerol and phosphorus (Smith, 1985). Each serotype appears to possess a distinctive lipoglycan and the surface location suggests that structural differences among lipoglycans could contribute to the antigenic specificity of the different serotypes (Smith, 1985).

Smith (1986) found that lipoglycans of varied carbohydrate composition associated with the membrane of *U. urealyticum* made up 5% of the cellular dry weight.

The significance of the ureaplasma lipoglycans in biological interactions is unknown, but it is unlikely that they are responsible for haemadsorption since only

serotype 3 exhibits this activity (Black, 1973b). This material may also influence phagocytosis as well as possibly influencing the ability of the ureaplasmas to adsorb to host epithelia. It is noteworthy that lipoglycans have not as yet been found on all Mollicutes.

1.7.5. ANTIBIOTIC RESISTANCE.

Strains of *U. urealyticum* demonstrate a differential response to antibiotics. Whilst most strain are sensitive to tetracyclines, they are resistant to, for example, rifampicin and trimethoprim (Davis and Hana, 1981). By 1976, however, 10% of isolates from N.G.U. were resistant to tetracycline (Evans and Taylor-Robinson, 1978). Strains recently isolated from N.G.U. patients by Taylor-Robinson and Furr (1986), showed similar levels of tetracycline resistance but approximately 40 % of these resistant strains are also resistant to erythromycin. Thus, whilst resistance levels are apparently not increasing, this may constitute a virulence factor in a minority of strains. Early reports suggested that resistance not only to tetracycline, but also to chloramphenicol and dihydrostreptomycin in several mycoplasmas, was due to a reduction in membrane permeability to the drugs (Maniloff and Morowitz, 1972). A recent study by Roberts and Kenny (1986) on serotypes 1-9 and on 63 clinical isolates with respect to tetracycline resistance, showed that 13 of the clinical isolates and serotype 9 were resistant to high levels of tetracycline suggesting that tetracycline resistance may be an inbuilt characteristic of certain serotypes only. The resistant isolates and serotype 9 all contained DNA sequences homologous to the streptococcal determinant tet M which suggests the spread of this gene to certain ureaplasmas. The tet M determinant appears to alter the organism's ribosomes, rendering them resistant to tetracycline and this then suggests an alternative resistance mechanism to membrane permeability.

More recently, Roberts *et al.* (1988) suggested that 20 % of *U. urealyticum* strains are now resistant to tetracycline. These workers also isolated a 4.9 Kb fragment from

U. urealyticum which contained the tetracycline resistance determinant Tet M which coded for a protein with a molecular weight of approximately 73 KDa and this protein shared 95 % homology with a Tet M gene from *Streptococcus pneumoniae*. This protein was thought to elicit resistance in one of two ways: (1) by inhibiting the binding of the tetracycline to the ribosome. (2) it may act as an elongation factor which is resistant to tetracycline.

Antibiotic resistance may allow *U. urealyticum* to persist in tissues, an ability which may be of significance outside the urinary tract where very high levels of antibiotics cannot be reached and thus chronic infections may result.

As *U. urealyticum* appears to be increasingly resistant to tetracyclines, Kenny and Cartwright (1988) have studied the effect of the newer quinolones on these organisms. Difloxacin and ofloxacin were the most inhibitory and may have distinct promise for the treatment of genital infections.

1.8. MOLECULAR BIOLOGY AND GENETICS OF MYCOPLASMAS (REVIEWED BY RAZIN, 1985).

The circular double stranded genome of Mollicutes is distinguished by its minute size and low guanine plus cytosine (G+C) content. The G+C content of most mycoplasmal DNA is between 25 -34 mol%, and with respect to *U. urealyticum*, is between 27-28 mol% (Black *et al.*, 1972a). The mycoplasmas fall into two clusters according to genome size: one composed of Mycoplasma and Ureaplasma species with a genome of approximately 5×10^8 daltons and the other composed of Acholeplasma, Spiroplasma and Anaeroplasm species with a genome of approximately 1×10^9 daltons. As the smallest known genome size is around 1×10^9 daltons, the Mycoplasma and Ureaplasma species have the smallest genome recorded for any self-reproducing prokaryotes (Bak *et al.*, 1969).

However, this 5×10^8 daltons may be an underestimate (Pyle *et al.*, 1988) as

pulse-field electrophoresis suggests that the genome is composed of 900 Kb rather than the 750 Kb which is equivalent to 5×10^8 daltons.

The extremely small size of mycoplasma and ureaplasma genome imposes several restrictions on coding capacity, explaining the low number of cell proteins since a genome size of 5×10^8 daltons is capable of coding for approximately 650 different proteins. As the bacterial genome usually contains spacers, including various signals that comprise 20-30 % of the total DNA, the actual numbers of genes would be around 500. Razin (1985) has detected over 300 proteins using 2-dimensional gel electrophoresis. Complex nutritional requirements suggest that most, if not all, of the genes for the metabolic pathway enzymes for producing building blocks of macromolecules are missing, but it is postulated that membrane systems for transport of these building blocks from the environment should exist in these organisms (Razin *et al.*, 1968). This would explain the complex nutritional requirements of these organisms.

It would appear that mycoplasmas arose as a branch of the low G+C Gram-positive tree near the Lactobacilli and Streptococci. The initial event in mycoplasma phylogeny appears to be the formation of the Achleoplasma branch; hence, loss of the cell wall probably occurred at the time of genome reduction to approximately 1000 MDa. A subsequent branch produced the Spiroplasma and this branch appears to have been the origin of sterol-requiring Mycoplasma species. During development of the Spiroplasma branch, there were several independent genome reductions, each to approximately 500 MDa, resulting in Mycoplasma and Ureaplasma species. (Rogers *et al.*, 1985).

Whilst the low G+C content is perhaps due to some mutation pressure replacing G.C pairs by A.T pairs, the nature of this mutation pressure is unknown at present. Work on *Mycoplasma capricolum* suggests that different parts of the genome contribute positively to the low G+C content of the genome. The spacer regions

between the genes are the lowest with respect to G+C content (about 20 %), the second lowest are the protein genes (about 30 %) and the G+C content of the rRNA and the tRNA coding regions are relatively high (47-54 %) (Muto *et al.*, 1987).

The mol% G+C values are effective tools in the classification of Mollicutes. A finding of a difference in the G+C content between bovine ureaplasmas (28.7-30.2 mol%) and the human *U. urealyticum* strains (26.9-28.0 mol%) served as an important indicator for the establishment of a new species (see section 1.1).

As mentioned before, the 14 serotypes of *U. urealyticum* form two distinct clusters as distinguished by electrophoretic patterns, cleavage patterns using restriction endonucleases (Razin *et al.*, 1983) and by exhibiting 40-60% DNA homology between the two clusters (Christiansen *et al.*, 1981). However, the G + C content does not differ between these two clusters and as yet the two clusters have not been classified as two separate species or even subspecies.

1.8.1. GENOME REPLICATION

Information on mycoplasma genome replication is still fragmentary. The replication of many Mollicutes resembles that of other prokaryotes in being semiconservative and proceeds sequentially from, at most a few replication forks. In *Acholeplasma laidlawii*, newly synthesised DNA, representing the growing point of the chromosome, appears to be membrane-associated (Smith and Hanawalt, 1968) and this observation was confirmed in *M. gallisepticum*. However, this latter organism has a bleb-infrableb structure and thus the situation may differ in the other mycoplasmas (Quinlan and Maniloff, 1973). In many mycoplasmas, cytoplasmic division lags behind genome replication, resulting in the formation of multi-nucleate filaments.

Three DNA polymerases have been isolated from Spiroplasmas and Acholeplasmas, but, as yet only one DNA polymerase has been found in *U. urealyticum* which lacks the 3'-5' exonuclease activity and thus raises the problem as to the proof reading

mechanism in these organisms (Buisson *et al.*, 1988).

There appears to be a wide occurrence of nucleases hydrolysing DNA and RNA which may play a role in the nutrition of these organisms by providing assimilable nucleic acid precursors from polymeric DNA and RNA (Razin, 1985). The endogenous nucleases may have an additional function such as DNA repair, restriction of foreign DNA and transport of transforming DNA. This rather intensive DNase activity greatly hampers the isolation of undegraded DNA and since the enzyme requires Mg^{2+} for activity, the use of EDTA helps prevent this digestion.

Mollicutes are very sensitive to ultraviolet and X- and gamma-ray irradiation. However both photoreactivation and dark repair mechanisms have been shown to operate in *Acholeplasma laidlawii* but are totally absent in *M. gallisepticum* and as yet, no information has been reported for *U. urealyticum*.

1.8.2. TRANSFORMATION AND TRANSFECTION.

The lack of a cell wall in Mollicutes would appear to favour genetic exchange by transformation, yet, despite many trials, very few have succeeded. These organisms are osmotically sensitive and tend to lose viability when suspended in buffer and this could account for the low success rate of transformation, but successful *in vitro* transformation of *A. laidlawii* and *M. pulmonis* with Tn 916 has been reported (Dybvig and Cassell, 1987).

One of the most recent successes of *in vivo* transformation has been with *Spiroplasma citri*, (Barroso and Labarere, 1988), where they have evidence of chromosomal transfer followed by recombination. The mechanism of gene transfer was insensitive to deoxyribonuclease, required contact between cells and possibly fusion between areas of the cell membranes. The potent nuclease activity of mycoplasmas as well as their tendency to lose viability in buffer solutions may also hinder transformation experiments (Razin, 1985). Transfection of mycoplasmas has met

with more success particularly with the aid of polyethylene glycol (PEG) (Sladeck and Maniloff, 1983) and many transfection systems have been reported (for a review see Razin, 1985). McCammon *et al.* (1988) have reported equally successful transfection frequencies with *S. citri* using 43 % PEG or electroporation.

1.8.3. TRANSCRIPTION.

1.8.3.1. RNA POLYMERASES.

The RNA polymerases of Mollicutes are insensitive to rifampicin, a property shared with RNA polymerases of Archaeobacteria and the subunit structure of these enzymes appears to resemble that of prokaryotes. Promoter and terminator regions in the few mycoplasmal species sequenced so far, also appear to resemble those of prokaryotes. Thus several genes that have been cloned from mycoplasmas can be expressed in *E. coli* cells, suggesting that the *E. coli* RNA polymerase recognises transcription signals of mycoplasmas (Mouches *et al.*, 1985).

1.8.3.2. RIBOSOMES

Mycoplasma ribosomes resemble typical prokaryotic ribosomes in having a sedimentation coefficient of about 70s, which dissociates into 30s and 50s subunits and one distinguishing property of these ribosomes is their sensitivity to low concentrations of divalent cations (Johnson and Harowitz, 1971). Mycoplasma ribosomes were dissociated into subunits in the presence of 5mM Mg^{2+} , whereas *E. coli* ribosomes remained intact. Three rRNA species (5s, 16s and 23s) have been identified, as have approximately 50 protein species which is again similar to the situation seen in other prokaryotes. However, the 16s RNAs from mycoplasmas examined so far, appear to be smaller in size than bacterial 16s RNAs by about 12000 daltons and a number of sequences which are normally highly conserved in bacteria appear to be absent from mycoplasmas (Neimark, 1984). 5s RNA sequences from three

mycoplasmas, *M. capricolum*, *M. mycoides subsp capri* and *Spiroplasma* sp BC3 have been published and each 5s is unusually short (Hori *et al.*, 1981; Walker *et al.*, 1982). Mollicutes have only one or two copies of rRNA genes; this low number is in line with the concept of economy in genetic information. *U. urealyticum* is thought to have 2 rRNA operons (Amikan, 1984; Ohse and Gobel, 1987) and the genes appear to be linked in the typical order 5'-16s-23s-5s-3'. However, the hybridisation patterns produced by the 14 serotypes showed differences which were most pronounced among serotypes belonging to the two different clusters (Razin and Yozegz, 1986). It has been reported (Chen and Finch, 1989) that the genome of *M. gallisepticum* contained 3 widely separated rRNA loci. One locus contained genes for all three rRNA species, another contained 23s and probably 5s rRNA genes, whereas the third appeared to have only a 16s rRNA gene.

Specific rRNA gene probes cloned from *M. capricolum* have been reported as being able to detect less than 1ng of mycoplasmal DNA which is roughly equivalent to the DNA content of 10^5 mycoplasmas. The possibility of using this approach for detection and identification of noncultivable mycoplasmas in plant and insect tissue is under investigation (Razin, 1984). *M. pneumoniae*-specific oligonucleotide probes complementary to variable regions in the 16s RNA have been used in dot blot assays to detect less than 1×10^3 mycoplasmas.

1.8.3.3. TRANSFER RNA (tRNA).

tRNA's, like rRNA's are highly conserved molecules with respect to size composition and function but the low G+C content of the mycoplasma genome is not reflected in the base composition of the mycoplasma tRNA's. The intragenic regions are very rich in A-T (over 80 mol%), while the genes themselves have a G+C content of about 55 mol%, considerably higher than the G+C content of the genome. Thus, the G+C values resemble those of prokaryotic tRNA's. The secondary structure also fits the familiar clover leaf model of tRNA's. There appears to be only enough DNA to

code for 44 different tRNA molecules in *M. capricolum*, (Tully *et al.*, 1974), whereas *E. coli* has enough DNA to code for 60-80 tRNA genes. Mycoplasmas also appear to lack many isoaccepting tRNA's species for several amino acids, again economising the amount of genetic information. Another interesting feature of mycoplasma tRNAs is the consistent lack of modified nucleosides which are found in other prokaryotes. This is also the case with mycoplasmal rRNAs (Johnson and Horowitz, 1971).

1.8.4. CODON USAGE.

Mycoplasmas appear to use A and U biased codons e.g. in *M. capricolum* UUA (leu), AUU (ile), and AGA (arg) are the most frequently used, while they are rarely used in *E. coli*, where CUC (leu), AUC (ile) and CGU (arg) are predominant. More than 90 % of *M. capricolum* codons have A or U at the third position, in contrast to only 49 % of the *E. coli* codons.

The codon UGA, which is normally a stop codon in both prokaryotes and eukaryotes, codes for tryptophan in *M. capricolum* (Yamao *et al.*, 1985) and this is also the case with *M. pneumoniae* and *M. genitalium* (Inamine *et al.*, 1988). This would explain why the translation of many mycoplasma proteins are terminated when cloned in *E. coli*. Whether the above is true for ureaplasmas is as yet unknown.

1.8.5. PLASMIDS AND VIRUSES.

The possible existence of viruses in mycoplasmas was reported by Edwards and Fogh (1960). The first mycoplasma virus was isolated and characterised by Gourlay (1970) and subsequently more than 50 viruses of different morphologies were isolated, mainly from strains of *Acholeplasma laidlawii* and Spiroplasmas, although some have been found in several Mycoplasma species (Cole, 1979).

Extrachromosomal DNA not associated with virus particles has also been reported and these 'plasmids' have been detected in *A. laidlawii*, *M. hominis* and *M. arthritis*

but do not appear to be present in *U. urealyticum*, serotypes 1-9 (Harasawa and Barile, 1983). They are approximately 20×10^6 daltons in size and an average of 50-100 plasmids per cell has been reported (Maniloff *et al.*, 1977). However, as yet, no function has been defined for these plasmids.

Viral and plasmid DNA is frequently integrated into the chromosome, leading to lysogeny in the case of viruses (Razin, 1985).

1.9. AIMS OF THIS RESEARCH.

Much of the research on *U. urealyticum* is still in its infancy and a lot of basic information on this organism is lacking.

U. urealyticum appears to play a part in many disease states but to what extent it is involved is still unknown due to the fact that many laboratories do not routinely test for ureaplasmas. This is because they are extremely fastidious and require a complex growth medium, which, as yet is not easily available. Thus any studies carried out on this organism are done only by specialised groups of workers.

Thus the main aims of this research are to further elucidate some of the molecular biology of this organism, particularly with respect to important antigenic polypeptides, and to produce diagnostic systems for the detection of *U. urealyticum* from human clinical samples which could be commercially developed. These kits would not only be less time consuming, but would also allow workers outwith this field to examine clinical samples for the presence of this organism.

CHAPTER 2
MATERIALS AND METHODS.

All chemicals used in this study were supplied by BDH unless otherwise stated.

2.1. ORGANISMS USED.

(a) *Ureaplasma urealyticum* strains: Serotypes 1 (T7), 4 (11860), 6 (12253) and 8 (T960) were gifts from Dr. D. Taylor-Robinson (C.R.C., Harrow, U.K.). Serotypes 2 (T23), 3 (DKF3), 5 (NIH 5), 9 (Vancouver), 10 (Western), 11 (11-JsL 2), 12 (12-JsL 5), 13 (13-JsL 6) and 14 (14-JsL 11) were gifts from Professor J. Robertson (University of Alberta, Edmonton, Canada). Serotype 7 (ATCC 27819) was obtained from the American Type Culture Collection.

(b) *Ureaplasma diversum* strains: Strains T44, 7860/1 and E172/1 were gifts from Dr. G. E. Jones (Moredun Institute, Edinburgh, Scotland).

(c) Non-human ureaplasmas: Ureaplasmas isolated from canines (9255), felines (8638), chimpanzees (13863, 13838, 13849, 13882, 13892) and marmosets (13583) were also gifts from Dr. D. Taylor-Robinson.

(d) *Mycoplasma hominis*. Strain WHO PG21 was also donated by Dr. D. Taylor-Robinson.

(e) *Mycoplasma ovipneumoniae*. Strain 956/2 was a gift from Miss R. K. Spooner (University of St Andrews, Scotland).

(f) *Proteus*, *Serratia* and *Klebsiella* strains:

Proven urease-positive strains of a *Proteus* species, *P. vulgaris*, *P. rettgeri*, *P. mirabilis*, *Serratia liquefaciens*, *Klebsiella aerogenes*, *K. pneumoniae* (M169), and *Klebsiella* species (SW35, SW85, SW87, ST44, and ST81 all isolated from above or below a sewer outfall) were gifts from Dr. D. Thirkell (University of St Andrews, Scotland).

2.2. MEDIUM AND CELL PRODUCTION.

Ureaplasma urealyticum, *Ureaplasma diversum* and the other non-human ureaplasmas:-

Except where stated, ureaplasmas were grown in medium containing 70 % (v/v) PPLO broth (Difco), 20 % (v/v) horse serum (NBL) and 2.5 % (w/v) fresh yeast extract, incorporating 0.1 % (w/v) urea, 0.005 % (w/v) phenol red and 1000 IU penicillin G ml⁻¹ (Glaxo), at a starting pH of 6.0. Cultures were incubated at 37° C until a pH of 7.6, corresponding to a cell density of 10⁷ c.c.u. ml⁻¹ was reached. Determination of colour-change units (c.c.u.) was performed by serial decimal dilutions of 0.2 ml cultures in vials containing 1.8 ml of growth medium which were incubated at 37° C until no further colour change of the phenol red indicator was apparent (normally 48h). From the highest dilution in which colour change was seen, the number of c.c.u. ml⁻¹ of the original inoculum was determined. The cells were harvested by centrifugation using a Beckman J-21 centrifuge (25000g, 20 min), the pellets washed in Dulbecco's phosphate buffered saline 'A' (PBS) (Dulbecco and Vogt, 1954) and the final pellet resuspended in PBS (1ml/ 2.5 litre culture). Unless used immediately, the pellet suspension was aliquoted (100 µl aliquots) and stored at -70° C.

Mycoplasma hominis:- This organism was grown as described above but substituting 0.2 % (w/v) arginine hydrochloride for 0.1 % (w/v) urea. Initial pH was 7.0 and the culture harvested as above when the external pH had reached 7.8. The pellet was resuspended in PBS as above and used immediately or aliquoted (50 µl) and stored at -20°C.

Mycoplasma ovipneumoniae:- Cells were grown in 20 % (v/v) Brain Heart Infusion (Oxoid), 6 % (v/v) 10 X Medium 199 (Gibco), 10 % (v/v) inactivated newborn calf serum (Sera-Lab), 10 % (v/v) freshly prepared yeast extract, 0.008 % (w/v) phenol red and 1 mg ml⁻¹ Penbritin (Beecham) at a starting pH of 7.6-7.8. Cultures were grown at 37°C

until a pH of 6.8–7.0 was reached corresponding to 5×10^8 c.c.u. ml^{-1} . Cells were then harvested and stored as described for *M. hominis*.

Proteus, Serratia and Klebsiella strains:- 20 ml cultures were grown overnight in nutrient broth (Oxoid) at 37°C and used immediately.

2.3. RADIOIMMUNE ASSAY (RIA).

Nitrocellulose paper was incubated (60 min, 37°C) in a suspension of *U. urealyticum*, serotype 8, prepared as in section 2.3.8, with a protein concentration of 10–100 $\mu\text{g ml}^{-1}$ (dot-blot assay). The paper was washed in PBS, blocked with 10 % (w/v) Marvel (Cadbury) in PBS (30 min), and then washed several times in PBS containing 0.2% (v/v) Nonidet P40 (NP40) (Sigma) (PBS-N). The paper was sandwiched between two 96-well microtitre plates (Cell Cult) with the lower plate containing the test antibodies e.g. from a mouse tail bleed or from media overlaying hybridoma cells. The 'sandwich' was then inverted, ensuring the test material was in contact with the paper, and incubated at 37°C for 1 h. The paper was then washed in PBS-N, followed by incubation at 20°C for a further 1h in 10–20ml PBS-N to which 50 μl of ^{125}I -labelled Protein A (Amersham) was added. For detection of immunoglobulins of classes other than Ig G, the paper was incubated (60 min, 37°C) with 10–20 ml of a 1/200 dilution of goat anti-mouse immunoglobulins (GAM) (Sigma) in PBS-N, before incubation in the radiolabelled Protein A. Finally the paper was washed repeatedly with several changes of PBS-N over a period of 30 minutes, dried and subjected to autoradiography (section 2.6.1).

2.4. ANTIBODY PRODUCTION.

(a) Production of Monoclonal Antibodies (Mab's):-

Monoclonal antibodies were raised from two separate fusions (Fusions A and B) and for a general review on methodology, see Goding, Ch 3, 1986.

Fusion A:- This fusion was carried out by Mrs P. Braddock as described below. Balb/C mice were immunised with *Ureaplasma urealyticum*, serotype 8, grown and harvested as described in section 2.2. Washed cells (approximately 10^7 c.c.u. ml^{-1}) on ice, were sonicated (4 X 10 second bursts with intermittent cooling) using a 0.5 cm microprobe on a Kerry ultrasonicator and then alum-precipitated (Mautner and Wilcox, 1974).

Initial immunisation (intraperitoneal, (ip)) was with 0.2 ml alum-precipitated cell extract followed by a second such injection 4 weeks later. A third injection (intravenous, (iv)) of 0.1 ml sonicated cell extract was given 4 weeks later, 3 days before the fusion. Mouse antiserum taken 2 days before fusion was screened by R.I.A. (section 2.3) for anti-ureaplasma antibodies using organisms grown in a medium with 10 % (v/v) new-born calf serum substituted for horse serum.

The spleen was removed from the mouse showing the greatest immune response and was gently homogenised in 5 ml PBS using a glass homogeniser in a boiling tube. After allowing particulate tissue material to settle, the overlying suspension containing the spleen cells was removed and centrifuged at 300 g for 5 min. Any red blood cells present were lysed by shaking in 0.83 % (w/v) ammonium chloride and removed from the spleen cells by centrifugation (300g, 5 min).

Mouse myeloma cells (SP2/0), frozen in liquid nitrogen in 1ml freezing mix (1 ml DMSO, 3 ml new born calf serum (Sera-Lab), 6 ml Glasgow's modified minimal Eagles medium (Gibco) (MEM)) were thawed at 37°C and centrifuged (300g, 5 min). The supernatant was discarded, 10 ml fresh MEM added and the cells recentrifuged (300g, 5 min). The cells were then resuspended in a further 10 ml MEM containing 10 % (v/v) new born calf serum (10 % MEM) and placed in 75 cm^3 tissue culture flats (Cell-Cult) at 37°C in an atmosphere of 5 % CO_2 , cell suspensions were prepared by first replacing the spent medium with fresh and the flats subjected to vigorous tapping.

Macrophages were prepared from sacrificed Balb/C mice by injecting 5ml of warm

MEM (ip). The abdomen of the mouse was then massaged gently for 2-3 min after which a second needle was inserted into the left-hand side of the abdomen and the fluid containing the macrophages removed. This fluid was centrifuged (300 g, 8 min) and any red blood cells present were removed as described above. The macrophages from one mouse were resuspended in 30 ml 10 % MEM and were used to seed 3 X 96-well plates (100 μ l/well).

Spleen and SP2/0 myeloma cells were counted on a haemocytometer slide and spleen cells (6.8×10^7) were mixed with SP2/0 myeloma cells (1.0×10^7) in a ratio of 2:1 with respect to cell numbers and giving a total volume of 6.5 ml, which was then made up to 50 ml in PBS. The cells were centrifuged (300g, 5 min), resuspended in 50 ml PBS and similarly centrifuged. The final cell pellet was resuspended in residual PBS by gentle tapping, 0.5 ml polyethylene glycol (PEG) added and cells were centrifuged at 300 g for 1-2 min to ensure close contact. These fused cells were incubated at 37°C for 20 seconds, slowly diluted with 20 ml 10 % MEM, and again centrifuged (300g, 5 min). The fused cells were gently resuspended in 5 ml 10 % MEM containing hypoxanthine, aminopterin and thymidine (HAT) (Sigma, 50X HAT), used at final concentration of 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine. A further 50 ml of 10 % MEM/HAT medium was added to the 5 ml of cells and six 96-well plates (containing 100 μ l macrophages/well) were seeded with 36 ml of this cell suspension (100 μ l/well). The remainder of the cells was diluted 1/2 in 10 % MEM/HAT and a further six 96-well plates also containing 100 μ l macrophages/well, were seeded with this cell suspension (100 μ l/well as above). Outer wells were filled with 10 % MEM/HAT medium only, to prevent the wells containing cells from drying out. Mouse SP2/0 myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase and cannot grow in minimal medium containing HAT. Although spleen cells do possess this enzyme they will also not survive in tissue culture medium for more than a few days as they are not immortal. Thus, unfused myeloma and spleen cells survive only a few days in HAT medium and only fused

spleen/myeloma cells (hybridoma cells) survive and grow. After 5-7 days, the HAT medium was replaced with HT medium (as HAT without the aminopterin) and after a further 14 days the cells were placed in 10 % MEM alone.

When the wells were 50 % confluent with hybridoma cells, the medium above the cells was tested for the presence of antibodies using RIA (section 2.3) and by enzyme-linked immunosorbent assay (ELISA) using a goat peroxidase-linked anti-mouse immunoglobulin (Sigma) as described by Precious et al. (1987). Positive clones were transferred to 24-well plates (previously seeded with macrophages) by resuspension in 200 μ l of fresh 10 % MEM, retested when 50 % confluent and clones which were positive at this stage, were dilution cloned.

Dilution cloning: Each positive clone was resuspended in 200 μ l of fresh 10% MEM when 80-100% confluent and the cells first diluted 1/1000 in 10 % MEM. From this cell suspension, three 5-fold dilutions were prepared in 10 % MEM (1/5000, 1/25000, 1/125000) and all four dilutions were plated out in 96-well plates (100 μ l/well) which had been previously seeded with macrophages as before.

After three days, each well was examined for the presence of a single cell and any wells containing more than one cell were eliminated. After six days, wells were re-examined for the presence of a single clone and only wells containing single clones were retested when 50 % confluency was reached.

Positive clones were then transferred (as above) to 24-well plates and retested twice and when 100 % confluent, were suspended in 500 μ l MEM, centrifuged (1000 g, 3 min) and resuspended in 500 μ l MEM. Pristane-treated Balb/C mice (0.5 ml pristane (Sigma) injected (ip) 5-7 days previously) were injected intraperitoneally with these cells and ascitic fluid removed usually between 8-14 days later. Any contaminating red blood cells were removed using 0.83 % (w/v) ammonium chloride and centrifuged as described previously, 0.1 % (w/v) sodium azide added and after aliquoting (500 μ l), stored at -20° C. Any hybridoma cells present were resuspended in 10 ml 10 % (v/v)

MEM and grown to 100 % confluency in 75 cm³ flats in an atmosphere of 5 % CO₂ . Spent medium was removed from the cells which were then resuspended in 10 ml MEM, centrifuged (300g, 5 min) and finally resuspended in 1 ml freezing mix and placed in 2 ml plastic vials. These vials were placed in an insulated box at -70°C for 2-3 days before being transferred to liquid nitrogen.

Fusion B:-

Using a previously obtained Mab (UU8/1) which recognised the urease enzyme of *U. urealyticum*, serotype 8, (Precious *et al*, 1987), purified urease was prepared using affinity chromatography and FPLC (section 2.11; 2.18). This urease was used either as the native enzyme or in the denatured state (boiling 2 min) as an immunogen to hyperimmunise two sets of six Balb/C mice following a protocol as described for fusion A.

Three days after the final iv injections of the urease preparations, the mice were tail-bled and using RIA, antibody levels against the urease were determined so that the spleens from the mice with the highest antibody titres could be used in the fusions.

Two fusions were carried out using standard techniques (as for Fusion A). Antibodies were tested by RIA as before and by using the urease catch test (section 2.21). However the fusion performed with the spleen from the mouse immunised with denatured urease was not successful. Only a small number of hybridomas grew and cells proved to be negative for the production of anti-urease antibodies by the above techniques. As the other fusion was producing large numbers of antibody-producing clones it was decided to continue with this one only.

(b) Production of Polyclonal serum:-

Polyclonal serum was produced as described by Precious *et al*, (1987).

Briefly, rabbits were injected (0.5 ml X 2) (intramuscularly) with 5×10^9 c.c.u. ml⁻¹ (grown in medium containing horse serum) with a ratio of 1:1 (with respect to volume) of Freund's complete adjuvant followed by a series of further intramuscular injections in complete adjuvant at 3 week intervals for 3 months. Animals were test bled periodically and antisera monitored by R.I.A. (section 2.3). Further booster injections were given intramuscularly at 3 month intervals and the animals bled for sera as and when required. The serum was aliquoted and stored at -20°C.

2.5. PROTEIN ESTIMATIONS.

These were carried out using three different methods as appropriate.

(a) Using the method of Bradford (1976) where an accurate measurement was required:-

A standard curve was prepared using a range of 10-100 µg ml⁻¹ of bovine serum albumin (BSA) (Sigma) diluted in PBS. For each reaction, 10 µl of protein solution was mixed with 1 ml of Bradford's reagent (0.01% (w/v) Coomassie Blue G-250 (Sigma), 8.8 % (w/v) phosphoric acid, filtered before use) and incubated at 20°C for 4-10 min. The absorbance (O.D.) was read at 595 nm within 1 h on a CECIL CE 272 spectrophotometer.

(b) Dot-Blots:-

Doubling dilutions of BSA containing 10 mg - 0.078 mg protein ml⁻¹ were prepared in PBS and 2 µl of each dilution were spotted on to nitrocellulose sheet. The sheet was then stained with 0.2 % (w/v) naphthalene black 12B in 10 % (v/v) acetic acid, 40 % (v/v) methanol for 3 min and destained in 7.5 % (v/v) acetic acid, 25 % (v/v) methanol. Protein samples were also subjected to doubling dilutions in PBS and an approximation of protein concentration was made by visual comparison of the two sets of dilutions.

(c) Absorbance:-

The absorbance of protein samples was read at 280 nm on a Perkin-Elmer Lambda 5 UV/Vis spectrophotometer, where 1 O.D. is approximately equivalent to a protein concentration of 1 mg ml^{-1} .

2.6. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE).

2.6.1. SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

Using 10 % or 15 % (w/v) polyacrylamide slab gels, the method of Russell and Blair (1977) was followed except where stated. Samples were prepared for electrophoresis, (i) by making to 25 % (v/v) with respect to glycerol and without any prior heat treatment, or (ii) by boiling for 2 min after addition of an equal volume of 'denaturing mix' (2.5M urea, 1.75M β -mercaptoethanol (Sigma), 1 % (w/v) SDS and 0.1 % (w/v) bromophenol blue). Staining of polypeptides was achieved either with Coomassie stain or with the more sensitive silver stain (Bio-Rad kit). For Coomassie staining, gels were fixed for 1 h in 10 % (w/v) trichloroacetic acid, 40 % (v/v) methanol, stained for 4 h in 0.3 % (w/v) PAGE blue 83, 46 % (v/v) methanol, 7.5 % (v/v) acetic acid (stain was filtered before use) and destained with 7.5 % (v/v) acetic acid and 25 % (v/v) methanol.

Where labelled material was electrophoresed, autoradiography was carried out as described by Russell and Blair (1977) using x-ray film (Fuji RX) and a Philips fast tungstate intensifying screen.

Apparent molecular masses were determined, where appropriate, by comparison with protein standards (Bethesda Research Laboratories).

2.6.2. PAGE IN ABSENCE OF SDS(NON-DENATURED PAGE).

Polyacrylamide gels (7.5 % or 10 %, w/v) were prepared as described in (a) above without the presence of SDS in either the gel or the buffer.

2.7. IMMUNOBLOTTING.

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets (Schleicher & Schuell) was carried out on an Ancos Model A semi-dry electroblotter according to the supplier's directions. Briefly, 6 sheets of chromatography paper (Whatmann No 1) was immersed in anode 1 buffer (0.3M Tris, 20 % (v/v) methanol, pH 10.4) and then placed on the lower anode graphite plate. Chromatography paper (3 sheets) were immersed in anode 2 buffer (0.025M Tris, 20 % (v/v) methanol, pH 10.4) and then placed on top followed by a nitrocellulose sheet (presoaked in distilled water) and the polyacrylamide gel. Finally, 6 sheets of chromatography paper immersed in cathode buffer (0.025M Tris, 40mM 6-amino-n-hexonic acid (Sigma), 20 % (v/v) methanol, pH 9.4) and then placed on top followed by the cathode graphite plate. Proteins were transferred at $0.8\text{mA}/\text{cm}^2$ of gel for 1 h. The nitrocellulose paper was then blocked with 3 % (w/v) BSA (Sigma) (30 min, 20°C), washed three times with PBS-N and either left as whole sheets or cut into strips. Strips were placed in a slotted plastic tray, each strip incubated (37°C , 2 h) with 2 ml of a 1/500 dilution (in 50:50 (v/v) PBS-N: growth medium) of an appropriate monoclonal antibody (Mab) or with hyperimmune homologous polyclonal serum (see section 2.4 (b)). After washing three times with PBS-N, the individual strips were probed with 2 ml of a 1/500 dilution (in PBS-N) of sheep anti-mouse immunoglobulin (Sigma) and incubated at 37°C for 2 h. Each strip was then washed and incubated at 20°C for 1h in 2 ml of PBS-N containing approximately 10^6cpm , ^{125}I -labelled Protein A (Amersham). Immunoglobulin G's (Ig G) were detected by screening with ^{125}I -labelled Protein A alone. The nitrocellulose strips were washed extensively in PBS-N, dried and

subjected to autoradiography using x-ray film,(Fugi RX) and a Philips fast tungstate intensifying screen.

When nitrocellulose sheets were not cut into strips, they were placed in a 'sandwich box' and processed as described above, using 2 ml of each appropriate solution per track on the original gels.

Alternatively, nitrocellulose strips were blocked with 10 % (w/v) Marvel in Tris-buffered saline (20 mM Tris/HCl pH 7.5, 500 mM NaCl) (TBS), (90 min, 37°C) and then incubated with 2 ml of a 1/500 dilution of appropriate Mabs in TBS containing 10 % (w/v) Marvel (3 h, 37°C). After thorough washing in TBS containing 0.05 % (v/v) NP40 each strip was incubated for a further 3 h at 37° C in 2 ml of a 1/200 dilution of a goat anti-mouse Ig G peroxidase-linked antibody (Scottish Antibody Production Unit) (SAPU) in TBS containing 10 % (w/v) Marvel. After washing three times in TBS, the strips were developed for up to 1 h in the dark in a solution of 60 mg 4-chloro-1-naphthol (Sigma) in 20 ml methanol and 80 ml TBS to which 60 µl 30 % (v/v) H₂O₂ was added immediately prior to use.

When nitrocellulose sheets were not cut into strips, they were placed in a 'sandwich box' and processed as described above, using 2 ml of each appropriate solution per track on the original gels.

2.8. COMPETITION ASSAYS.

2.8.1. PURIFICATION OF MONOCLONAL ANTIBODIES (MABS).

Mabs of immunoglobulin classes other than Ig G were purified by ammonium sulphate precipitation. To 1 ml of an appropriate undiluted ascitic fluid, 100 % (w/v) saturated ammonium sulphate was added dropwise with stirring to a final concentration of 40 % (v/v). This solution was incubated overnight at 4°C, centrifuged (1000g, 5 min) and the precipitate resuspended in 200 µl PBS, dialysed overnight (4°C)

against PBS and used immediately or stored at 4°C.

Mabs of immunoglobulin G class were purified on protein A-Sepharose (Sigma) which was reswollen in PBS and prepared as a 1 ml column (Goswami and Russell, 1983). The ascitic fluid (1 ml) was passed through the column, which was then thoroughly washed with PBS-N until no more protein washed through (assayed by dot-blot on nitrocellulose which were then stained with 0.2 % (w/v) naphthalene black as described in section 2.5 (b)). The purified Mab was eluted from the protein A-Sepharose column using 0.1 M glycine, pH 2.8 (detected as above) and collected in 0.5 ml aliquots in tubes containing 200 µl MTris-HCl, pH 7.6. Pooled fractions were dialysed overnight against PBS at 4°C and used immediately or stored at 4°C.

2.8.2. RADIOLABELLING OF THE MONOCLONAL ANTIBODIES.

Appropriate purified antibodies were radiolabelled with ^{125}I by the method of Hunter (1978). Briefly, 20 µl purified Mab (approximately 5 mg protein ml⁻¹ assayed by 'dot-blot'; previously dialysed against PBS) was added to 20 µl of 0.02 % (w/v) chloramine-T in PBS and 500 µCi ^{125}I -labelled sodium iodide (100 mCi ml⁻¹) was added and after 2 min incubation at 20°C, 20 µl of 0.03 % (w/v) sodium metabisulphite was added to terminate the reaction. Excess radiolabel was removed by centrifugation (300g, 3 min) of the Mab through a 1 ml coarse Sephadex G-50 (reswollen in PBS) (Pharmacia) column prepared in a 1 ml syringe.

2.8.3. TITRATION OF LABELLED MONOCLONAL ANTIBODIES.

A 5 litre culture was grown as described in section 2.2 and the pellet resuspended in 3 mls of PBS. This concentrated cell suspension was sonicated (3 X 10 sec bursts with intermittent cooling on ice) using a 0.5 cm microprobe on a Kerry ultrasonicator and the cell extract aliquoted (100 µl) and used immediately or stored at -70°C. Nitrocellulose paper (8 X 5 cm²) was incubated (60 min, 20°C) in 10 ml PBS containing

50–100 $\mu\text{g protein ml}^{-1}$ (determined by 'dot-blots', section 2.5 (b)) from the sonicated cell extract. The nitrocellulose paper was blocked with 10 % (w/v) Marvel in PBS and washed three times in PBS-N. Doubling dilutions of radiolabelled antibodies (1/100 to 1/102,400 in PBS containing 1% (w/v) Marvel; final volume of 10 $\mu\text{l/well}$) were prepared in wells of an 84-well mini plate (Sterilin). The nitrocellulose sheet was placed over the 84-well plate containing the antibody dilutions and a 'sandwich' made by clipping a second inverted 84-well plate on top. After inversion to allow contact of the antibodies with the nitrocellulose sheet, the 'sandwich' was incubated at 20°C for 1 h (Randall *et al.*, 1987). The nitrocellulose was then washed extensively with PBS-N, dried and subjected to autoradiography as described in section 2.6.1. Labelled antibodies were used at concentrations equivalent to 8–10 times the titration end-point, as seen on overnight exposure of the autoradiogram (usually 1/3200 diluted in 1 % (w/v) Marvel in PBS).

2.8.4. ASSAY PROCEDURES.

Unlabelled antibodies were then tested for their ability to compete with the radiolabelled antibodies by making 4-fold dilutions of ascitic fluid in an appropriate dilution of labelled antibody (prepared in 84-well mini plates (Sterilin) in 15 μl volumes starting at a 1/20 dilution). Nitrocellulose sheet coated as in section 2.8.3, was placed over the 84-well plate and a 'sandwich' was prepared and incubated for 1 h at 20°C as before. The nitrocellulose sheet was washed extensively with PBS-N, dried and subjected to autoradiography as described in section 2.6.1.

In addition, a second competition assay involving the 'urease catch test', (section 2.21), was used to evaluate the epitopes recognised by the anti-urease Mabs. Wells of a 96-well plate were coated with GAM as described and after washing were coated with 100 μl of a selected Mab (1/100 dilution in PBS). After incubation (3 h, 37°C), washing with PBS, blocking with 3 % (w/v) BSA (30 min, 37°C) and a further wash with PBS, 100 μl of preincubated diluted antibody-ureaplasma mixtures (100 μl ureaplasma

cells in log phase diluted with an equal volume of PBS-N containing test antibody at final dilutions from 1/50-1/6400 and incubated for 1 h at 37° C) were added to each well of separate rows and the plate incubated for 1 h at 37° C. After incubation, washing once with PBS-N and three times with PBS, and addition of 100 µl 15 mM deionised urea to each well, the plate was incubated for 40 min at 20°C. To each well, 50 µl phenol nitroprusside and 50 µl alkaline hypochlorite were added and after 30 min at 20°C, colour development was read at 600nm on a Multiscan.

2.9. INHIBITION OF METABOLISM WITH ANTI-96 kDa AND WITH ANTI-16/17 kDa MABS.

Paper ELISAs were carried out to establish the immunoglobulin content of each monoclonal ascitic fluid such that similar concentrations of antibody could be compared in these metabolic inhibition studies. Doubling dilutions of appropriate ascitic fluids (1/50 - 1/6400) in PBS were placed in wells of an 84-well mini plate. Nitrocellulose paper which had been previously immersed in PBS was incubated (1 h, 37° C) with these dilutions in the form of a sandwich as described earlier. The paper was blocked with 3 % (w/v) BSA (30 min, 20°C) and washed three times with PBS. The nitrocellulose paper was then incubated (1 h, 37°C) with a 1/350 dilution of Goat anti-mouse peroxidase-linked immunoglobulin (Sigma) in PBS. After extensive washing in PBS, the paper was developed in the dark (30 min, 20°C). The developer was 150 mg O-dianisidine (Sigma) dissolved in 1 ml methanol and then diluted to 50 mls with distilled water. Any undissolved o-dianisidine was removed by filtration and the filtrate was made 0.06 % (v/v) with respect to H₂O₂ just before use.

Each Mab being tested was added to 50 ml culture medium at an appropriate dilution (usually at a final concentration of 1/50 or 1/100). The culture medium was inoculated with *U. urealyticum*, serotype 8, to give 10²c.c.u. ml⁻¹. Homologous hyperimmune polyclonal serum (see section 2.4 (b)), at a final concentration of 1/500, and an antiviral Mab (gifted by Dr. R. Randall, University of St Andrews, Scotland), at a final dilution of 1/1000, were used as positive and negative controls respectively. The

cultures were incubated for 26 h at 37°C and 3.5 ml aliquots were removed at times 0,9,12,17,19,24 & 26 h to assess cell growth and metabolism by detecting urease activity (using the urease catch test, section 2.21), pH of the external medium and determination of c.c.u. ml⁻¹.

2.10. PURIFICATION OF 96, 17, 16 kDa MEMBRANE ANTIGENS FROM UREAPLASMA UREALYTICUM, SEROTYPE 8.

2.10.1. PREPARATION OF AFFINITY COLUMNS (Axen *et al*, 1967).

1g of CNBr-activated Sepharose 4b (Sigma) was washed and reswollen in 200 ml 1mM HCl in a scintered glass funnel. The sepharose on the scintered surface was then washed with a total volume of 5-10 ml coupling buffer (0.1M NaHCO₃ pH 8.3 containing 0.5M NaCl). The appropriate Mab of immunoglobulin class G (1 ml), purified using a Protein A-Sepharose column, (section 2.8.1), was added to 10 ml of coupling buffer, and mixed overnight with the sepharose gel in an 'end-over-end' mixer at 4°C. Any remaining active groups on the gel were blocked using 20 ml 0.2M NaHCO₃, 0.1M NaCl, pH 8.0 containing 1M ethanolamine, by incubation for 2 h at 20°C. The excess of uncoupled antibodies and blocking agent was removed from the gel by washing alternatively with 0.1M acetate buffer, pH 4.0, and coupling buffer pH 8.3 (50-60 ml total volume of each buffer) with the activated sepharose on the scintered glass funnel surface. This process was repeated several times, after which the gel was placed in a 2 ml disposable syringe, washed with PBS-N and the column stored at 4°C.

2.10.2. PURIFICATION OF THE 96 kDa MEMBRANE ANTIGEN.

Cells from a 5 litre culture (grown and harvested as described in section 2.2), were resuspended in 2 ml PBS-N, sonicated as before (section 2.8), centrifuged (1000 g, 5 min) and the supernatant applied to the affinity column. The column was then washed

with PBS-N until no more protein washed through (assayed by dot-blots on nitrocellulose paper which was then stained with naphthalene black). The purified antigen was eluted using 0.1M glycine, pH 2.8 (detected as above) and collected in 0.5 ml fractions in tubes containing 200 μ l M Tris-HCl, pH 7.6, and the pooled fractions dialysed overnight against PBS at 4°C, aliquoted (500 μ l) and stored at -20°C.

2.10.3. PURIFICATION OF THE 16, 17 kDa ANTIGENS.

Cells from a 5 litre culture were processed as described in 2.10.2. However, pooled fractions from the column contained a variety of proteins, none of which appeared to be of molecular weights 16 or 17 kDa (assayed by SDS-PAGE, silver staining and immunoblotting). The original solubilised extract was also assayed as above, with the results suggesting the absence of these membrane antigens in the solubilised extract.

Thus, cells from a 5 litre culture (grown and harvested as previously described—section 2.2) were sonicated (3 X 10₃ secs on ice) in a buffer containing 10 mM Tris-HCl, pH 7.2, 5mM di-sodium ethylenediamine tetraacetate (EDTA), 0.5 % (v/v) NP40, 0.65M NaCl, 0.1 % (w/v) NaN₃, 1mM PMSF and 0.1% (w/v) SDS (Buffer A). This sonicated extract was centrifuged (1000g, 5 min) and the supernatant subjected to SDS-PAGE and immunoblotting. The supernatant was found to contain the 16/17 kDa antigens and was therefore passed down the affinity column as described in 2.10.2. However, the column was washed in the above buffer rather than PBS-N and the fractions were eluted and treated as described in 2.10.2 above.

2.11. PURIFICATION OF UREASE FROM SEROTYPE 8.

2.11.1. PREPARATION OF AFFINITY COLUMN.

Using an anti-urease Mab (Ig G), the affinity column was prepared as described in 2.10.1.

2.11.2. PURIFICATION (Following methodology previously described by Precious *et al.*, 1987).

The total cell pellet from a 10 litre culture of *U. urealyticum* was resuspended in 4 ml PBS-N and sonicated as described in section 2.8.3. After centrifugation (MSE microfuge, 1000 g, 5 min), the supernatant was removed and retained and the extraction repeated several times. Pooled supernatants were applied to the affinity column which was then washed with PBS-N as described 2.10.2; the urease was eluted with 0.1M borate buffer, pH 10.0 and the pooled fractions were dialysed overnight at 4°C against PBS. The purified urease was usually used immediately, but any surplus was stored at -70°C in the presence or absence of 50 % (v/v) glycerol in PBS and in siliconised or non-siliconised tubes.

2.12. DETECTION OF GLYCOPROTEINS (Knapp, P., 1978).

A cell suspension (section 2.8.3) or purified or partially purified antigen (section 2.10) was subjected to SDS-PAGE (section 2.6) and the electrophoresed polypeptides were transferred to nitrocellulose paper, (as described in section 2.7), which was then blocked with 3 % (w/v) BSA and washed three times in 50mM Tris pH 7.5, 100mM NaCl, 1mM MnCl₂, 1mM CaCl₂. The paper was then incubated at 37°C for 1h in 20 ml of the above buffer containing 1 µg ml⁻¹ peroxidase-linked concanavalin A (Sigma). After extensive washing with the same buffer, the paper was developed for up to 30 min in the dark in a solution of 60 mg 4-chloro-1-naphthol (Sigma) in 20 ml methanol and 80 ml PBS to which 6 µl H₂O₂ was added immediately prior to use. Influenza virus which has three major glycosylated components in denatured gel systems (haemagglutinin 1 and 2 and neuraminidase), (Wilson *et al.*, 1981; Varghese *et al.*, 1983), was used as a positive control.

2.13. PHASE PARTITIONING OF THE INTEGRAL MEMBRANE AND CYTOSOLIC PROTEINS OF UREAPLASMA UREALYTICUM, SEROTYPES 1, 7 AND 8 USING TRITON X-114, (TX-114).

Precondensation of TX-114:- 1 litre of 2 % (w/v) TX-114 (Sigma) in Buffer B (10 mM Tris-HCl pH 7.4, 150 mM NaCl) was incubated at 0°C for 10 min. This solution was then incubated at 30°C until two separate phases could be seen (usually 8-12 hrs). The larger aqueous phase was discarded and replaced with the same volume of fresh buffer B with mixing and this process was repeated 3 times resulting in up to 50 % loss of detergent.

Phase partitioning was carried out as described by Bordier (1981), utilising the modification of Reithman *et al.* (1987). Briefly, the protein samples, usually sonicated cell extracts, (0.2 - 1.0 mg protein ml⁻¹), were prepared in 200 µl of 10mM Tris-HCl, pH 7.4, 150mM NaCl and 1% (v/v) precondensed TX-114, and incubated on ice for 10 min. At this stage any unsolubilised proteins were removed by centrifugation at 2500g for 3 min.

For the separation of the solubilised protein, a cushion of 6 % (w/v) sucrose in the above buffer containing 0.01 % (v/v) TX-114 was placed at the bottom of an Eppendorf tube. The clear solubilised protein sample was then overlaid on this sucrose cushion and the tube was incubated at 30°C for 3 min during which clouding occurred. The tube was centrifuged for 3 min at 300g, after which the detergent phase was found as an oily droplet at the bottom of the tube. The upper aqueous phase was removed, made 0.5 % (v/v) with respect to TX-114 and the phase partition repeated. The final aqueous phase was rinsed with 2 % (v/v) TX-114 and the resultant detergent phase was discarded. Using TX-114 and buffer, both final detergent and aqueous phases were made to the same volume (200 µl) and to 1 % (v/v) with respect to TX-114

Aliquots of both phases were boiled in an equal volume of sample 'denaturing mix' for 2 min and then subjected to SDS-PAGE followed by silver staining (Bio-Rad) or immunoblotting and autoradiography.

2.14. LABELLING OF UREAPLASMA UREALYTICUM, SEROTYPE 8, WITH ^{125}I -LABELLED BOLTON AND HUNTER REAGENT (N-succinimidyl 3-(4-hydroxy-5-iodophenyl)propionate).

Fresh cultures (1 litre) were grown in medium containing 10% (v/v) foetal calf serum rather than 20% (v/v) horse serum as it was found that this reduced medium contamination of cell pellets (Precious *et al.*, 1987). The cells were centrifuged, (25000g, 20 min, 4°C), and the pellets resuspended in PBS and centrifuged as above. This procedure was repeated twice followed by resuspension of the pellets in 45 ml 0.1M sodium borate buffer, pH 8.3, before centrifugation was repeated and the final pellets were resuspended in 200 μl 0.1M borate buffer at approximately 10^4 c.c.u. ml^{-1} . The cell suspension (0.2 ml) was then labelled for 15 min with 100 μCi ^{125}I -labelled Bolton and Hunter reagent (Amersham) at 4°C, according to the supplier's instructions and the reaction stopped by the addition of 200 μl 0.1M glycine in 0.1M borate buffer, pH 8.3. The cells were washed three times, by centrifugation (1200g, 5 min) and resuspension in PBS, to remove excess label and any labelled internal components released by autolysis. The labelled cells were finally resuspended in 400 μl PBS and stored at -20°C. Sonicated cell extract (400 μl) was also labelled as described above. However, excess label was removed by passing the extract down a Sephadex G-50 (coarse) (Pharmacia) column.

2.15. IMMUNOPRECIPITATIONS.

Bolton and Hunter-labelled whole cells, or cellular extracts, were lysed in immune precipitation buffer, (10mM Tris-HCl pH 7.2, 5mM EDTA, 0.5% (v/v) NP40, 0.65M NaCl, 0.1% (w/v) NaN_3 , 1mM PMSF) and sonicated with an ultrasonic probe as described

previously (2.8.3); soluble labelled antigens were obtained by centrifugation (1200 g, 10 min) and the pellet was stored at -20°C.

Immune complexes were formed by incubating 50 µl samples of the soluble antigen with 5 µl of appropriate undiluted ascitic fluid for 1h on ice. The immune complexes were isolated on an excess of a fixed suspension (Kessler, 1975) of the Cowan A strain of *Staphylococcus aureus* (20 µl of a 10% (w/v) suspension /µl of ascitic fluid) for 1h at 4°C. The immune complexes of *S. aureus* were pelleted (1200g, 10 min), resuspended in 1 ml immunoprecipitation buffer containing 10 % (w/v) sucrose and centrifuged as above. Resuspension of the pellet in this buffer and centrifugation were repeated three times after which the final pellet was resuspended in 100 µl "denature mix" and boiled (3 min). After further centrifugation (as above), the supernatant was subjected to SDS-PAGE and following electrophoresis, the gels were either stained using the Bio-Rad silver staining kit and dried or dried without prior silver staining. Labelled polypeptides were visualised by autoradiography with Fuji X-ray film as before.

2.16. LABELLING OF UREAPLASMA UREALYTICUM, SEROTYPE 8, WITH (9,10(n)-³H) PALMITIC ACID.

Labelling was carried out in two ways:- In the first, a 100 ml culture was grown to early logarithmic-phase and harvested as described previously. The cells were then resuspended in 3 ml PBS, 0.5 % (v/v) horse serum to which 500 µCi (9,10(n)-³H) palmitic acid (50 Ci/mM) was added. The cell suspension was then incubated at 37°C for 2 h after which time, the cells were centrifuged (25000g, 20 min), the pellet resuspended in a further 100 ml of growth medium, and the cells finally harvested when the pH of the medium had reached 7.6. This cell pellet, resuspended in 200 µl 'denaturing mix' was boiled for 3 min, then subjected to SDS-PAGE and fluorography. For fluorography, the electrophoresed gels were fixed in 10 % (v/v) acetic acid, 40 % (v/v) methanol for 20 min, washed in 50 ml of dimethylsulphoxide (DMSO) (3 X 30 min)

and soaked for 3 h in 22 % (w/v) 2,5-dimethyloxazole (PPO) in DMSO. The gel was then placed under cold running water for 30 min, dried under vacuum (40°C, 2 hr) and exposed to X-ray film (Kodak, fast film) at -70°C for three months.

In the second method, cells were labelled with (9,10(n)-³H) palmitic acid (50 Ci/mM) by growth in 100 ml medium containing 1.25 % (v/v) horse serum, 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES buffer) (Sigma) and 500 µCi of the labelled fatty acid at inoculation. Ureaplasmas were harvested as described previously when the pH of the medium had risen to 7.1 and the cells were subjected to either immunoprecipitation or phase separation using TX-114 (section 2.13) and then SDS-PAGE and fluorography. This second method proved to be the more successful and was used in all subsequent experiments.

2.17. DETECTION OF UREASE ACTIVITY ON GELS AFTER SDS-PAGE OR AFTER NON-DENATURING PAGE.

10 % (w/v) polyacrylamide gels were prepared as in section 2.6.1 and 2.6.2. These gels were loaded with freshly prepared purified urease (15 µg protein per track; assayed by the method of Bradford (1976) or with a sonicated cell extract of ureaplasma cells (section 2.8.3) (50 µg protein per track; Bradford, 1976), both made to 25 % (v/v) glycerol, without any prior heat treatment. After electrophoresis, the gels were stained in two ways. However, those gels containing SDS were pre-washed in PBS-N (3 X 30 min).

(1) Fishbein stain (Fishbein, W.N., 1969):-

The gels were shaken in 0.05M citrate buffer, pH 6.0, (3 X 100 ml) for a total of 1-2 h and after removal of the buffer, the gels were stained for 20-30 min with the following solution- 3.6 ml 0.5M citrate buffer, pH 6.0, 5.2 ml M urea, 5.0 ml p-nitro blue tetrazolium (0.5 % w/v) (Sigma), 14.0 ml 15 mM dithiothreitol (Sigma) and made to 66 ml with distilled water. The regions on the gel containing urease activity were

visible as purple/blue bands.

(2) Lead acetate stain (Mahaboob *et al.*, 1980):-

The gels were shaken for 20 min in 100 ml 50 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA, shaken for a further 20 min in 20 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA and then incubated in 1 mM EDTA containing 1.5 % (w/v) urea for 3 min. The gels were rinsed in distilled water and incubated in 100 ml of 0.1 M lead acetate after which the regions on the gel containing urease activity were visible as white opaque bands.

2.18. DETERMINATION OF THE MOLECULAR MASS OF IMMUNOAFFINITY PURIFIED UREASE OF SEROTYPE 8 BY GEL FILTRATION (FPLC).

An FPLC instrument (Pharmacia) was used with a 30 cm Superose 6 column using 280 nm protein detection on an LKB BROMMA spectrophotometer. Elution was carried out with PBS at a flow rate of 0.25 ml min^{-1} at 20°C with a pressure of 1.0 MPa. The column was calibrated by applying a mixture of molecular weight standards within the range 12kDa to 640kDa (Sigma) under the same conditions. 200 μl of freshly prepared enzyme (in PBS) was applied to the column and 0.75 ml aliquots collected (flow rate 0.25 ml min^{-1}). All fractions were assayed for urease activity by the Bertholot reaction and, having noted the elution volume, the molecular mass of the enzyme was determined.

2.19. UREASE SUB-UNIT RATIO BY DENSITOMETRY.

After fully denatured enzyme (prepared as in section 2.6.1) had been subjected to SDS-PAGE on 15 % (w/v) polyacrylamide gels, these were stained with Coomassie blue and subjected to densitometry using a Vitatron densitometer. The individual subunit peak areas were calculated and on the basis of established molecular masses, the molar ratios of individual subunits established.

2.20. pH ACTIVITY OF THE UREASE FROM UREAPLASMA UREALYTICUM, SEROTYPE 8.

The pH activity of this enzyme was evaluated over a wide range of pH (pH 2.2–10.7) by Frost (Thirkell *et al.*, 1989). In this investigation, a narrower pH range was used to verify the actual pH optimum of the enzyme. The activity of aliquots of freshly prepared enzyme, purified by immunoaffinity chromatography, was measured in a 0.1M citrate buffer over a final pH range of 5.0–8.1 with the pH readjusted after the addition of 15 mM urea. A 1/10 dilution of the urease preparation in PBS was then incubated in the above buffers at 20°C for 40 min after which 25 μ l phenol nitroprusside and 25 μ l alkaline hypochlorite were added and colour development was read at 600nm on a Multiscan after 30 min at 20°C.

2.21. UREASE CATCH TEST.

This was a modification of the test reported by Precious *et al.* (1987). Each well of a 96 well microtitre plate (Cel-Cult) was pre-coated with 100 μ l of a 1/200 dilution of goat anti-mouse immunoglobulins (GAM) (Sigma) in PBS by incubation overnight at 4°C. After removal of the GAM, the wells of the plate were washed once with PBS and then 100 μ l of 1/100 dilution of an anti-urease Mab in PBS was added to each well and the plate incubated at 37°C for 3 hr. Wells of the plate were washed twice with PBS and then each well was 'blocked' by the addition of 200 μ l of 3 % (w/v) BSA and incubation for 30 min at 37°C. Wells of the plate were again washed twice in PBS and then to each well, 100 μ l of a *U. urealyticum* culture (10^6 – 10^7 cells ml^{-1}) or a clinical sample (section 2.25), diluted 1/2 in 0.5 % (v/v) NP40 in PBS (PBS-N"), was added. The plate was incubated at 37°C for 1h, washed once in PBS-N" and then four times in PBS. 100 μ l of 15 mM deionised urea was added to each well and the plate incubated at 20°C for 30 min. 50 μ l of phenol nitroprusside and 50 μ l of alkaline hypochlorite (Sigma) (Bertholot reaction reagents) were added to each well and incubation of the plate continued for a further 30 min. A blue colour indicated the

presence of active urease enzyme and the colour intensity could be read on a Multiscan (Flow) at 600nm.

2.21.1. SENSITIVITY OF THE CATCH TEST.

A 20 ml culture of *U. urealyticum*, serotype 8, grown as described in section 2.2 was incubated at 37°C until a pH of 7.6 was reached. 0.2 ml was removed and the c.c.u. ml⁻¹ was determined (section 2.2).

From this 20 ml culture, duplicate 10 fold dilutions were prepared in PBS-N" and 100 µl aliquots were subjected to the above catch test assay. The colour intensity was read on a Multiscan at 600nm and the lowest number of organisms detectable was calculated.

2.22. ELECTRON MICROSCOPY, THE ULTRASTRUCTURE OF *U. UREALYTICUM*

Cells in a 500 ml culture of *Ureaplasma urealyticum*, serotype 8, grown as described in section 2.2, were prefixed for 15 min at 4°C after the addition of glutaraldehyde (Sigma) to a final concentration of 2.5 % (v/v). The fixed cells were pelleted (25000g, 20 min) and post-fixed in 2 ml of 1 % (w/v) osmium tetroxide in PBS for 1 h at 20°C. After removal of the osmium tetroxide, the fixed cells were rinsed in PBS, dehydrated using 5 ml aliquots of an ascending acetone series (50 %, 70 %, 90 %, 100 % (v/v); 15 min for each concentration of acetone) and then mixed overnight in 4 ml of 50 % (v/v) araldite (Taab)(prepared as directed by the supplier) in 100 % acetone on a rotory mixer at 20°C. The araldite-acetone was then replaced with 100 % araldite from which all the air had previously been removed under vacuum, and aliquots were placed in conical beam capsules (Agar Aids) with the cell pellet at the base. The resin was then polymerised at 60°C for 24 h, after which gold sections were cut using a Huxley ultramicrotome and collected on copper grids (Emscope, 300 nm).

Grids were stained for 5 min in Reynolds lead citrate, washed in distilled water for

30 seconds and counterstained in 2 % (w/v) aqueous uranyl acetate.

Examination of initial sections on a Phillips electron microscope (Model EM 301) revealed the presence of an unacceptable amount of amorphous material which may have been due to aggregated medium components or the inability to adequately fix medium contaminated cells. Significant medium contamination of cell pellets from cultures grown in horse serum-containing medium can be greatly reduced if the horse serum is substituted with 10 % (v/v) foetal calf serum (NBL) (Precious *et al.*, 1987). As a consequence the above procedure was repeated using cells grown in a medium containing 10 % (v/v) foetal calf serum.

2.23. IMMUNOCYTOCHEMISTRY.

Cultures of *Ureaplasma urealyticum* serotype 8 (500 ml) were grown in medium containing 10 % foetal calf serum (section 2.22) and the organisms fixed in two ways:- In the first, cells were pelleted as in section 2.2, resuspended in 40 ml 0.5 % (v/v) glutaraldehyde in PBS, incubated for 2 h at 4°C and repelleted as before.

In the second, three cultures grown as above were pre-fixed by addition of an equal volume of 1 % (v/v) glutaraldehyde in PBS and incubated at 4°C for 30 min or 1 or 2 h before centrifugation of fixed cells (25000g, 20 min). These pellets, in glass vials, were then dehydrated through an ascending ethanol series (30 %, 50 %, 70 % (v/v),; 15 min each concentration of ethanol) followed by three changes (15 min each) in 70 % (v/v) ethanol. After removal of the 70 % (v/v) ethanol, the pellets were rotated in 4 ml LR white resin (Medium)(Taab) (LR White):70 % (v/v) ethanol:: 1:3 (v/v) for 1 h at 20°C. The LR White/ethanol was then replaced with LR White resin alone and the pellets were similarly rotated overnight. The pellets were then rinsed in three changes of LR White before being placed at the base of gelatin capsules (Parke, Davis & Co) which were then filled with LR White resin to form a meniscus. These were then polymerised at 50°C in the absence of air (achieved by placing an inverted gelatin

capsule lid on top of the filled capsule) for 24 h. Gold sections were cut using a Huxley ultramicrotome from the cells which had been fixed for 30 min only, prior to embedding (cells fixed for 1 and 2 h were not subsequently examined). These sections were collected in water on nickel grids (Emscope, 300nm).

Grids were incubated overnight, in petri dishes, at 4°C or at 37°C for 1 h on 40 µl droplets of purified Ig G anti-urease Mab (UU8/17) and anti-96kDa Mab (UU8/29) diluted in Tris-HCl, pH 8.2, containing 20 mM NaN₃, 225 mM NaCl and 0.1% (w/v) BSA (neat, 1/5, 1/10, 1/50, 1/100). The grids were washed several times in the above buffer and then incubated on 20 µl droplets of Protein A Gold, (10 nm), (Biocell), at a dilution of 1/10 in the above buffer, for 1h at 20°C. The grids were then thoroughly washed in distilled water and stained with lead citrate and uranyl acetate as described above.

All sections were examined using a Phillips EM301 electron microscope.

The results seen using the anti-96kDa Mab (UU8/29) were poor, thus a third method was used, which involved the growth of a 400 ml culture of *U. urealyticum*, serotype 8, in 10 % (v/v) foetal calf serum which was grown and harvested as described (section 2.2). The cell pellet was resuspended in 2 ml PBS to which 1 ml of an anti-96kDa Mab (UU8/29) ascitic fluid was added and incubation was carried out for 2 h on ice. The pellet was washed 3 X in PBS, 1 X in Tris-HCl, pH 8.2 (as described above), by centrifugation and resuspension (50 ml). The cell pellet was then resuspended in 2 ml Tris buffer; at this stage 1 ml of the suspension was fixed for 30 min at 4°C in an equal volume of 1 % (v/v) glutaraldehyde in PBS, dehydrated and embedded in LR White as described previously. Sections were cut and stained with Protein A gold as described above.

Protein A gold (1 ml of a 1/10 dilution in Tris buffer) was incubated with the remaining 1 ml of cell suspension for 1 h at 20°C with shaking, after which time the cells were washed 3 X in PBS as described above, and the cell pellet was fixed in 2.5 % (v/v) glutaraldehyde in PBS for 15 min at 4°C. The cells were recentrifuged and the

cell pellet was dehydrated, embedded in LR White, sectioned and examined as described above.

2.24. IMMUNOFLUORESCENCE.

The general methodology followed throughout has been described by Goding, Ch 7, (1986).

An anti-urease Mab (UU8/17), an anti-96kDa Mab (UU8/29), and an anti-16/17kDa Mab (UU8/39), all of which were of immunoglobulin class G, were purified using a 1 ml protein A-Sepharose column as described in section 2.8. In addition an anti-urease Mab (UU8/1) (Precious *et al.*, 1987), which was not of immunoglobulin class G was purified by ammonium sulphate precipitation (section 2.8).

A 10 litre batch of *U. urealyticum*, serotype 8, was cultured and harvested as described in section 2.2. The pellet was resuspended in 5 ml PBS and doubling dilutions of this, ranging from 1/40-1/320 in PBS, were prepared. 5 μ l volumes of these diluted antigen suspensions were air dried on separate wells of a Hendley and Essex (H&E) slide. The antigen was then fixed by immersing the slide for 5 min in 5 % (v/v) formaldehyde and 2 % (w/v) sucrose in PBS after which the slide was washed thoroughly in PBS containing 1 % (v/v) horse serum. Permeabilisation was carried out by immersing the slide in 10 % (w/v) sucrose in PBS-N for 5 min, followed by washing as above with a final wash in ultrapure water and excess water was removed without allowing the antigen to dry out.

2.24.1. INDIRECT METHOD.

This technique used rhodamine-linked anti-mouse antibody (Sigma). 5 μ l volumes of purified Mab (approximately 5-10 mg proteins ml⁻¹, as assayed with dot-blots) were incubated with the fixed antigens on the slide in a damp box for 1 h at 37°C. The antigen-antibody complex was permeabilised and washed as described

above. 5 μ l of the rhodamine-linked antibody were incubated with each complex again using the damp box. The slide was permeabilised and washed thoroughly as before, after which the slide was viewed on a fluorescent microscope (Jenalumar, Model SH250) at 570 nm, using a 276 barrier filter. *M. hominis* and *M. ovipneumoniae*, grown and harvested as described in section 2.2 were treated as described in 2.24 above and used as negative controls. Photographs were taken using a Kodak colour film (200 ASA) or a Kodak black and white film (400 ASA).

2.24.2. DIRECT METHOD.

Fluorescein isothiocyanate (FITC),(Sigma) was used as the fluorochrome. Purified antibody (1 ml) was dialysed overnight against 0.2 M NaHCO₃, 0.081 M Na₂CO₃, pH 9.5 and the absorbance (O.D.) at 280 nm of the dialysate was utilised to calculate the approximate protein concentration (section 2.5 (c)). A stock solution of FITC (1 mg ml⁻¹) in DMSO was prepared. The volume of this required to be added to the antibody solution (dropwise) and the incubation conditions were determined on the basis of the information shown in Table 1 (see Goding, 1986, Table 7.3, p259).

Table 1. Suggested conditions for labelling antibodies with FITC.

Protein concentration (mg/ml)	Mass of Fluorochrome (μ g/mg protein)	Ideal absorbance ratio	Conditions
10	10-20	495:280=0.5-1.0	pH 9.5, 20 °C, 2hr
3	50	495:280=0.5-1.0	pH 9.5, 20 °C, 2hr
1	100	495:280=0.5-1.0	pH 9.5, 20 °C, 2hr

The figures given in this table are guidelines only.

Any unreacted or hydrolyzed dye was removed by gel filtration utilising Sephadex G-25 (medium) (Pharmacia). The first coloured fraction to emerge (containing the conjugated protein) was collected in 0.5 ml aliquots and the absorbance of each aliquot determined at 280 nm and at 495 nm, using a Perkin-Elmer Lambda 5 spectrophotometer, and the fluorochrome to protein ratio (F:P) of these aliquots was then calculated as follows:-

$$F:P = \frac{2.87 \times \text{O.D. } 495 \text{ nm}}{\text{O.D. } 280 \text{ nm} - 0.35 \times \text{O.D. } 495 \text{ nm}}$$

Optimally, FITC-conjugated antibodies should have a molar F/P ratio of approximately 2-3 for use with fixed cells and where the F/P ratio was too high i.e. above 3, the fraction was passed down a second Sephadex G-25 column. Normally the first fraction collected had an F/P ratio within the optimal range.

The conjugate with optimal F/P ratio was incubated with the antigen as described in 2.24. *Mycoplasma ovipneumoniae* and *Mycoplasma hominis*, grown and harvested as described in section 2.2, were used as control antigens at a cellular protein concentration similar to that of the ureaplasma antigen (usually at a starting concentration of 5 mg protein ml⁻¹).

2.24.3. SENSITIVITY OF THE FITC-CONJUGATED MAB'S.

The sensitivity of the fluorescent probes was studied in two ways:- (i) Dilutions from a 20 ml culture of *U. urealyticum*, serotype 8, (1/10 dilutions in PBS from 10^7 to 10^1 c.c.u. ml^{-1}), were air dried and fixed on (H & E) slides and probed with FITC-conjugated Mab's as in 2.24 above.

(ii) Monolayers of baby hamster kidney (BHK) cells were grown in 75 cm^3 tissue culture flats in 10 % (v/v) MEM (Section 2.4(a)) containing 10 % (v/v) tryptose phosphate broth. Similarly, monolayers of HeLa cells were grown except that 10 % (v/v) MEM alone was used. For removal of the cells from the flats, the medium was aspirated and approximately 20 ml trypsin (0.05 % (w/v)) containing 0.02 % (w/v) EDTA in PBS, pH 7.2 was added. After incubation at 37°C for 3-5 min, this solution was discarded, 10 ml of medium added and the flats vigorously tapped to dislodge the cells into suspension. The cell density was evaluated using a haemocytometer slide and the volume adjusted to give a cell density of 2.7×10^4 $100 \mu\text{l}^{-1}$. Separate wells on a H & E slide were spotted with 75 μl of this cell suspension, the slide placed in a 15 cm^2 plastic box with lid (Sterilin) and incubated to confluence overnight at 37°C in an atmosphere of 5% CO_2 .

Any remaining medium was removed and 50 μl dilutions (in growth medium) of *U. urealyticum*, serotype 8 (corresponding to 10^7 - 10^1 c.c.u. ml^{-1}) were allowed to adsorb to the cells for varying times (30, 60, 90, 120 min) at 37°C . After these times, the slides were washed in PBS to remove suspended organisms, fixed in 5 % (v/v) formaldehyde and then treated as in 2.24 above using the FITC-labelled antibody. The slides were then viewed at 510 nm using a 247 barrier filter and photographs were taken as described in 2.24.1 above.

2.25. CLINICAL SAMPLES.

(a) Clinical samples were obtained, in duplicate, from the urethras of 91 male patients attending the genitourinary clinic at the Royal Infirmary, Dundee over a 3 month period. These were in the form of calcium alginate swabs, which after sampling, were placed in 1 ml of PBS and frozen at -70°C or were placed in Stuart's transport medium and stored at 4°C until processed (on same day or up to 3 days later).

The swabs placed in Stuart's transport media were processed by being streaked out on solid media containing 70 % (v/v) PPLO agar, 10 % (v/v) boiled blood extract, 10 % (v/v) Fleischman's yeast extract, 10% (v/v) pig serum, incorporating 0.002 % (w/v) phenol red, 0.1 % (w/v) urea, 0.03 % (w/v) manganese sulphate, 0.01 % (w/v) thallos acetate and 200 IU ml^{-1} of penicillin at a starting pH of 6.0. The plates were incubated in a candle jar at 37°C for 5-6 days without prior drying. This was done by Francis Miller at Ninewells Hospital, Dundee.

The swabs in PBS which had been stored at -70°C were thawed, and dissolved as much as possible in the PBS. Samples (50 μl) were removed, diluted with 50 μl of PBS-N" and assayed using the urease catch test (section 2.21).

After 50 such samples were assayed it was decided to freeze the swabs in 20 % (v/v) glycerol in PBS. These were processed as described above.

(b) Urine samples were taken from male patients attending the Praed Street clinic, St Mary's Hospital, London and titres of ureaplasma were assayed from each sample soon after voiding. The remainder of each of the urine samples was stored at -70°C and supplied by Dr. D. Taylor-Robinson, (CRC, Harrow). After thawing they were subjected to the protocol as shown below.

Protocol for frozen urine samples, thawed at 37°C:

(1) 200 μ l of sample was added to 1.8 ml growth medium containing 0.05 % (w/v) thallos acetate (clinical growth medium) giving a 10^{-1} dilution. Subsequent 10^{-2} , 10^{-3} and 10^{-4} dilutions were prepared similarly and all were incubated at 37°C for titration of c.c.u. ml^{-1} .

(2) 100 μ l was used directly in the 'enzyme catch test'.

(3) 50 μ l was diluted with an equal volume of PBS-N" and this was used in the 'catch test'.

(4) The remainder of the urine was centrifuged (300 g, 5 min):

(i) The pellet was resuspended in 500 μ l PBS:-

(a) 200 μ l of the suspension was added to 1.8 ml clinical growth medium (10^{-1} dilution). Subsequent 10^{-2} , 10^{-3} and 10^{-4} dilutions were prepared and all were incubated at 37°C for titration of c.c.u. ml^{-1} .

(b) 50 μ l of the suspension was diluted with an equal volume of PBS-N" and used in the 'catch test'.

(ii) Supernatant:-

(a) 200 μ l of supernatant was added to 1.8 ml clinical growth medium (10^{-1} dilution) and diluted further as above. All dilutions were incubated at 37°C for titration of c.c.u. ml^{-1} .

(b) 50 μ l of supernatant was diluted with an equal volume of PBS-N" and used in the 'catch test'.

Residual pellet and supernatant were combined, the pH was checked (in range 6.0–7.8) and the suspension made 0.5 % (v/v) with respect to NP40. This solution was

then tested using the dip stick (section 2.26) method.

Positive controls of *U. urealyticum*, serotype 8 were used throughout.

2.25.1. CONTROL INVESTIGATIONS.

(a) A seed culture of *U. urealyticum*, serotype 8 was diluted in clinical growth medium to give 10^4 , 10^3 , 10^2 and 10^1 c.c.u. ml⁻¹. These dilutions were frozen at -70°C for 2 days and after thawing, the viable titres remaining were established by titration as normal.

(b) The seed culture was also diluted in urine as above and the protocol described in (a) repeated.

(c) Both the dilution series ((a) and (b)) were assayed using the catch test.

2.26. THE DETECTION OF UREASE FROM UREAPLASMAS USING A 'DIP STICK'.

Squares of nitrocellulose paper (1 cm²) were incubated in a 1/50 dilution in PBS of an anti-urease Mab overnight at 4°C. The squares were washed in PBS, blocked with 3 % (w/v) BSA in PBS (30 min, 20°C, with shaking) and then washed twice in PBS and stored at 4°C in PBS.

Seeds of *Ureaplasma urealyticum* (10^5 c.c.u. ml⁻¹) or clinical samples (Section 2.25) were made to 0.25 % (v/v) with respect to NP40. Squares of nitrocellulose, treated as described above, were incubated in these samples (200 µl) at 37°C for 3 h with shaking. The squares were removed and washed once with PBS-N", three times with PBS alone and then incubated in 200 µl 15 mM urea in PBS for 40 min at 20°C in a glass vial. 100 µl of phenol nitroprusside and 100 µl of alkaline hypochlorite were added and colour development was read at 600nm on a multiscan after 30 min by transferring 200 µl of this solution to a 96-well plate.

2.26.1. SENSITIVITY OF THE 'DIP STICK'.

The sensitivity of this test was studied in two ways:-

(1) As for the catch test (section 2.21), colour development was read with the multiscan as described above.

(2) The nitrocellulose squares were incubated in 10 mls of PBS-N containing 100 μ l dilutions of a log culture of *U. urealyticum*. The nitrocellulose squares were then processed as described previously and colour development read using the multiscan as above.

2.27. SLIDE AGGLUTINATION.

Three solid supports were utilised:-

(1) A 10% (v/v) suspension of heat fixed *Staph aureus* (Cowan strain A, donated by Mr J. Smart, Stobhill Hospital, Glasgow), stored at 4°C, was used. To 1ml of this suspension, 100 μ l of anti-96 kDa Mab (UU8/29) or of anti-16/17 kDa Mab (UU8/39) or of anti-urease Mab (UU8/17), all of immunoglobulin class G and all previously purified, was added and incubated for 1h at 20°C. The immune complexes were pelleted at 1200g for 3 min on a microfuge. The supernatant was removed and the pellet resuspended in 1ml of 0.2 % (w/v) methylene blue in distilled water and further incubated at 20°C for 30 min. The complex was again centrifuged, the supernatant removed and the pellet resuspended in 10 ml PBS containing 0.1 % (w/v) NaN₃.

(2) A 1 % (w/v) suspension of 0.246 μ m diameter latex beads (Metachem Diagnostics Ltd) in 0.1M glycine in PBS, pH 8.2, was used. An equal volume of 500 μ l purified anti-16/17 kDa Ig G monoclonal antibody (UU8/39), at dilutions of 1/10 and 1/100 in PBS, was incubated with the latex beads for 2 h at 37°C. The complex was pelleted as described above and resuspended in 1 ml of 0.1M glycine buffer, pH 8.2,

containing 0.1 % (w/v) BSA. Anti-96 kDa and anti-urease Mabs were similarly treated.

(3) Tocyl-activated Dynospheres (Dyna) (XP6505, 10.4 μm diameter and XP-6501, 3 μm in diameter), were used as the third support. Purified anti-96 kDa, Mab (UU8/29) or purified anti-16/17 kDa Mab (UU8/39) (0.25 ml; 1 mg protein ml^{-1}) in phosphate buffered saline (0.01 M phosphate, 0.15 M sodium chloride), pH 7.5 was incubated with 25 mg dynospheres in 0.4 M boric acid, pH 9.5 for 16–20 h at 20°C in an 'end over end' mixer. The particles were washed by sequential washing with 1 ml of the following solutions:(1) 1M ethanolamine containing 0.1 % (v/v) Tween 20, pH 9.5, (2) 0.05 M Tris containing 0.1 M NaCl, 0.1 % (w/v) BSA, 0.0 1% (w/v) sodium azide, 0.1 % (v/v) Tween 20 (Sigma), pH 7.5, (3) Buffer (2) without Tween 20. Each washing was performed by 'end-over-end' rotation for 4–20 h at 20°C. Finally, the particles were suspended in 1 ml of buffer (3) and stored at 4°C.

Method:- 100 μl of each antibody-support complex was mixed on a glass slide with 100 μl of a *U. urealyticum* (serotype 8) cell suspension (grown and harvested as in section 2.2; 0.5 mg protein ml^{-1} ; assayed by dot-blots). Each complex was also mixed with PBS, growth medium and with *M. hominis* (grown and harvested as in section 2.2 0.5 mg protein ml^{-1}) as negative controls.

CHAPTER 3
THREE MEMBRANE ANTIGENS OF *UREAPLASMA UREALYTICUM* SEROTYPE 8.

As discussed in Chapter 1, (section 1.6) the pathogenicity of *Ureaplasma urealyticum* may be associated with certain serotypes or groups of serotypes only. This suggests that there are differences between the fourteen serotypes, either in terms of survival and ability to grow and reproduce in the host, or in terms of associated virulence factors (section 1.7). Probes which will recognise and differentiate the fourteen serotypes of *U. urealyticum* would obviously assist in investigations to determine whether the above is correct.

In this study, monoclonal antibodies (Mabs) were raised against serotype 8 (type strain) (section 2.4), in order to study the predominant antigens of this organism and of this serotype in particular.

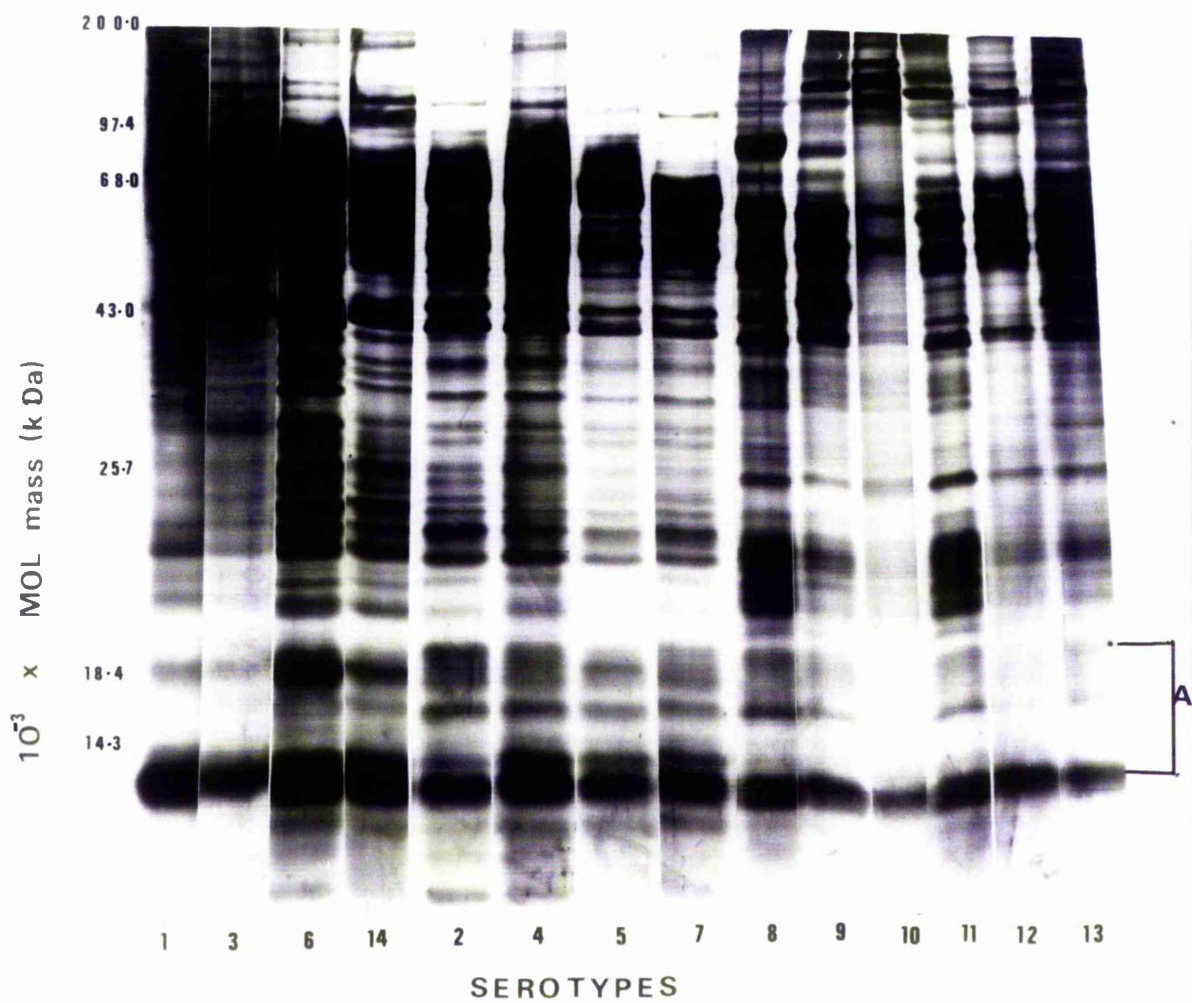
3.1. THE SEROTYPES OF *U. UREALYTICUM*

At present there are 14 recognised serotypes of *U. urealyticum* which can be divided into two seroclusters by a variety of techniques (section 1.1).

3.1.1. ELECTROPHORETIC ANALYSIS OF THE SEROTYPES.

All 14 serotypes, grown in a medium with 10 % (v/v) foetal calf serum substituted for horse serum, were analysed by SDS-PAGE and visualised either with the Coomassie blue stain or with the more sensitive silver stain. Whilst the latter reveals those polypeptide bands present in minimal quantity and every effort was made to have equal loading on each track of the gel, it was difficult to achieve a perfect separation with all 14 serotypes on one gel. Nevertheless, as seen in Fig 3.1, the polypeptide patterns of all 14 serotypes are very complex and show remarkable similarity to each other. Some minor differences in polypeptides and major quantitative differences in individual polypeptides are apparent but these differences were not considered to be sufficiently striking to justify either serotyping or differentiation into seroclusters by this single dimension technique. However, those

Fig 3.1: *U. urealyticum*, serotypes 1-14, subjected to 15 % (w/v) SDS-PAGE and silver staining (approximately 50 µg protein per track).



serotypes belonging to the smaller serocluster (cluster B) did appear to have fewer polypeptides in region A (Fig 3.1) than did those serotypes belonging to the larger serocluster (cluster A).

3.2. THE 96 kDa ANTIGEN.

Ten Mabs, designated UU8/29-38, were produced which recognised a 96kDa antigen in serotype 8 by SDS-PAGE (section 2.6) and immunoblotting (section 2.7). Nine of these Mabs were of immunoglobulin class G (Ig G), as detected using ¹²⁵I-labelled Protein A which binds to the Fc portion of Ig G antibodies with very high affinity (Forsgen and Sjoquist, 1966; Kronvall and Williams, 1969). The reactivity of Protein A towards different immunoglobulin classes and subclasses of different animal species has been reviewed by Lindmark *et al.* (1983). Fig 3.2 (lanes A-E) shows the immunoblot results with five of these Mabs and lane F shows that this antigen is also recognised by homologous polyclonal serum, prepared as described in section 2.4 (b). The fact that so many Mabs were raised against this polypeptide suggests that the 96kDa antigen is a major antigen of serotype 8, and this would confirm the earlier report by Sayed and Kenny (1980).

3.2.1. COMPETITION ASSAYS.

Using purified radiolabelled Mabs (section 2.8), competition assays were carried out with these ten Mabs and with two other anti-96kDa Mabs (UU8/4-5) prepared from a previous fusion (Precious *et al.*, 1987). These competition assays revealed the presence of a minimum of four distinct epitopes, recognised as shown in table 3.1 below:-

Fig 3.2: Immunoblot of *U. urealyticum*, serotype 8 probed with anti-96 kDa
Mabs (UU8/29-33) (lanes A-E) and with homologous polyclonal antiserum (lane
F) and ^{125}I -labelled Protein A after electrophoretic separation on a 15 % (w/v)
SDS-polyacrylamide gel (~ 100 μg protein per track).

A
B
C
D
E
F

96 kDa



polyclonal
serum

Table 3.1: Epitopes on the 96 kDa antigen recognised by individual Mabs.

Epitope	Mabs recognising the epitope.
1	UU8/29, UU8/33, (UU8/34-36, UU8/30)▲
2	UU8/4, UU8/5
3	UU8/37, UU8/38, UU8/31
4	UU8/34-36, UU8/30, UU8/32

▲ Steric hindrance (see text) i.e. may represent a separate group.

The results of two such assays, using ^{125}I -labelled UU8/29 and UU8/5 are shown in Fig 3.3. Using UU8/29, competition is evident in Row A (homologous unlabelled antibody) and Row E, suggesting that these two Mabs recognise the same epitope. Competition to some extent, is also seen in Rows B,F,G and H, but this competition could not be easily diluted out, suggesting that the epitopes recognised by these two groups of Mabs are closely situated and that steric hindrance is evident in these three rows. Using Mab UU8/5, competition is seen in Rows A (homologous unlabelled antibody), and B, suggesting that these two Mabs recognise the same epitope.

3.2.2. INHIBITION OF METABOLISM BY ANTI-96 kDa MABS.

Metabolic Inhibition studies (section 2.9) were performed using one anti-96 kDa Mab from each of the four epitope groups. Fig 3.4,(a)/(b) shows the results of two such assays using (a) Mab UU8/29 and (b), Mab UU8/5. Each of the anti-96 kDa Mabs appeared to suppress the growth of serotype 8 for approximately eight hours as shown by suppression of a rise in external pH and by the levels of urease activity which is expressed in terms of ammonia production (detected using the urease catch assay, section 2.21). No such inhibition could be detected using a Mab against a viral antigen (as a negative control) at similar immunoglobulin concentrations. Homologous polyclonal serum completely inhibited the growth of serotype 8, whereas the inhibition

Fig 3.3: Competition assays with ^{125}I -labelled anti-96 kDa Mabs and other unlabelled Mabs.

4-fold dilutions of unlabelled antibody, 1/20-1/81920.

A: Competition against homologous unlabelled antibody.

B-L: Competition against other unlabelled anti-96 kDa Mabs.

**4-FOLD DILUTIONS OF UNLABELLED ANTIBODY
IN APPROPRIATE DILUTIONS OF LABELLED
ANTIBODY**

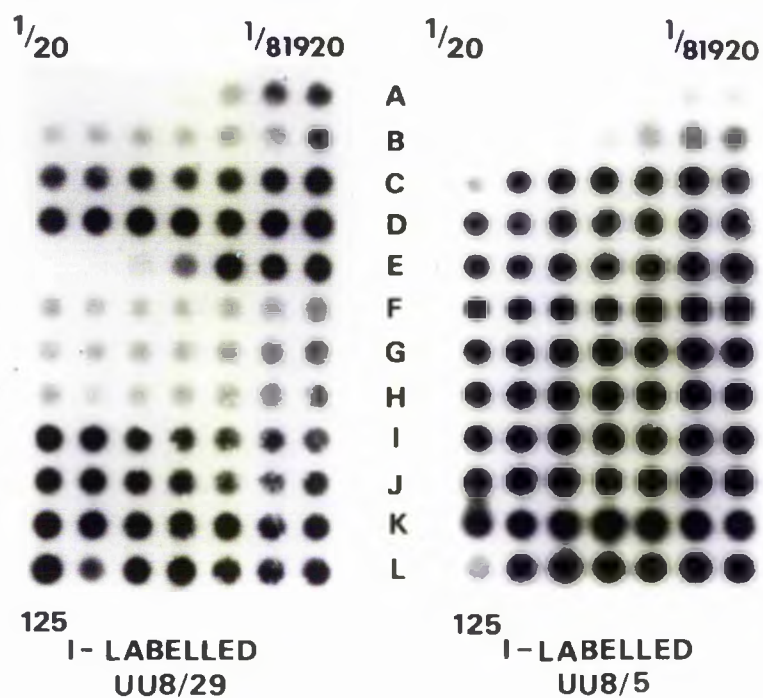
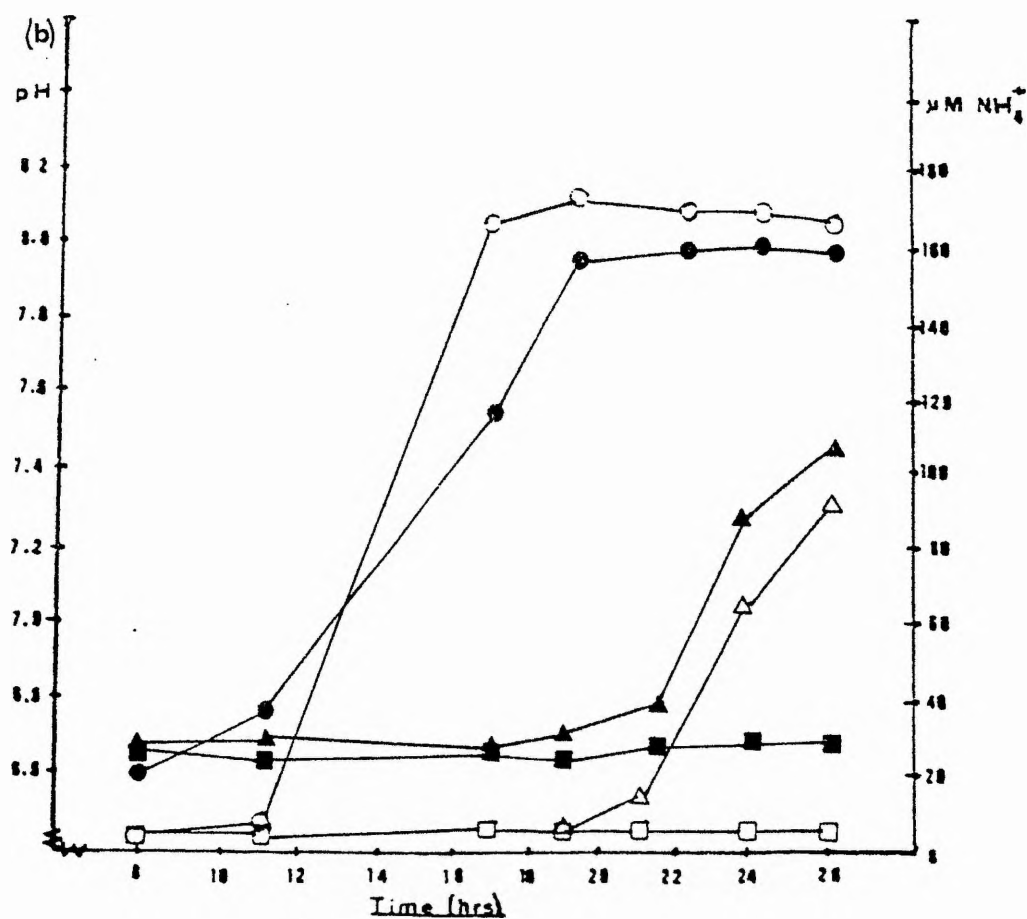
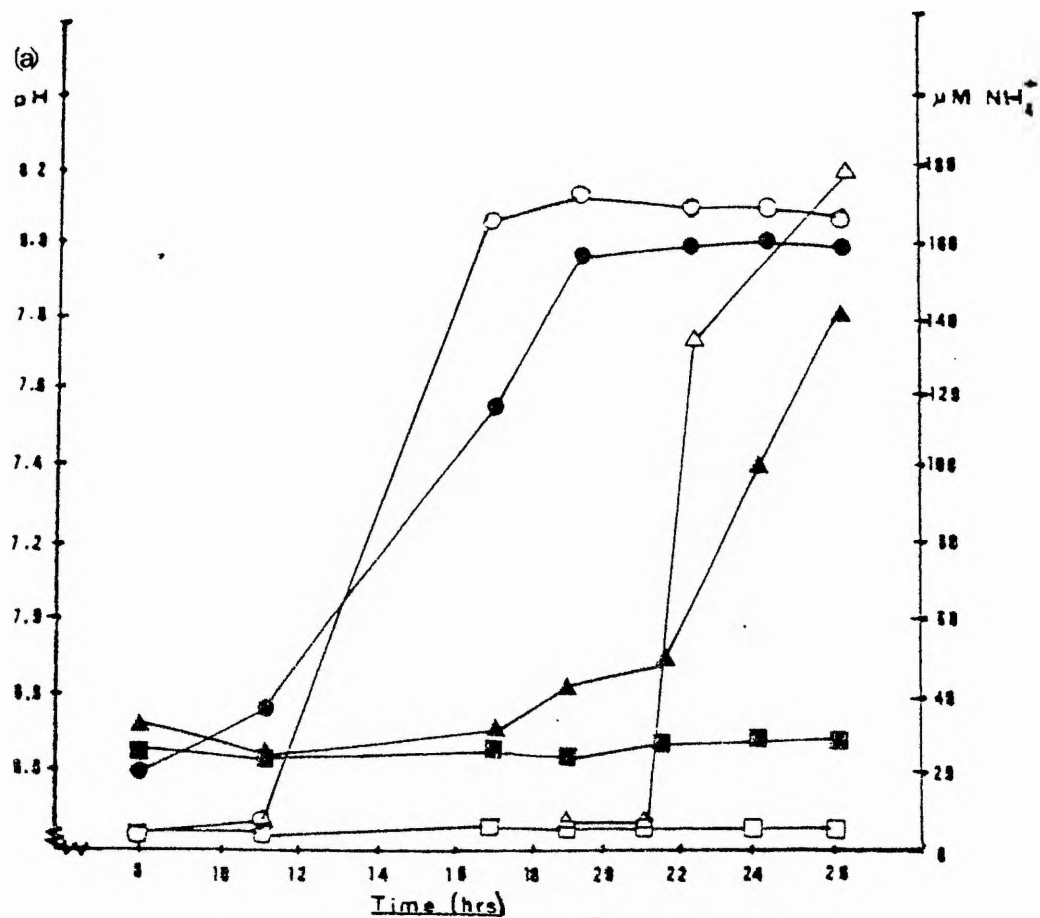


Fig 3.4: Inhibition of metabolic activity of *U. urealyticum*, serotype 8 by

(a) anti-96 kDa Mab UU8/29 and (b) anti-96 kDa Mab UU8/5. The organism was diluted to give a starting titre of approximately 10^2 c.c.u. ml^{-2} and incubated in the presence or absence of antibody. Samples were taken at various time points for pH measurements and for the estimation of urease activity ($\mu\text{M NH}_4^+$).

●, pH in the absence of antibody; ○, urease activity in the absence of antibody; ,
 ▲ pH, in the presence of (a) 1/100 Mab UU8/29 or (b) 1/100 Mab UU8/5; △ urease
 activity in the presence of (a) 1/100 Mab UU8/29 or (b) 1/100 Mab UU8/5; ■ pH
 in the presence of 1/100 homologous polyclonal rabbit anti-serum; □ urease
 activity in the presence of 1/100 homologous polyclonal antiserum.



seen with the anti-96 kDa Mabs was eventually overcome. Similar experiments were carried out using serotypes 7 and 1, and although the polyclonal serum against serotype 8 showed some inhibitory effect on serotype 7 but not on serotype 1, the Mab UU8/5 had, no inhibitory effect on either (Precious *et al*, 1987). The simultaneous addition of two or more Mabs from each of the four epitope groups gave no evidence of synergistic action as shown in Fig 3.5.

3.2.3. PURIFICATION OF THE 96kDa ANTIGEN OF SEROTYPE 8.

Using immuno-affinity chromatography with immobilised Mab UU8/29 (section 2.10), it was repeatedly shown that the 96 kDa antigen could only be partially purified. Despite thorough washing of the column with PBS-N (Fig 3.6, lane C), a contaminating polypeptide was always co-purified with the 96 kDa antigen as shown with Coomassie blue staining after SDS-PAGE (Fig 3.6, lane D). This contaminating polypeptide with an apparent molecular mass of 85 kDa, also co-purified when Mab UU8/5 was used on the immuno-affinity column (Precious, 1987, Personal Communication). Nevertheless, this 85 kDa polypeptide was not recognised by any of the anti-96 kDa Mabs as shown by SDS-PAGE and immunoblotting (Fig 3.7, lane A), but that it may be a glycoprotein was suggested by the fact that it stained with peroxidase-linked concanavalin A (section 2.12), (Fig 3.7, lane B). Conversely, the 96 kDa antigen did not stain with peroxidase-linked concanavalin A, suggesting that the 96 kDa antigen is not modified with glucose or mannose residues which are detected by that lectin. This does not rule out the possibility that the 96 kDa antigen may be modified with a more unusual sugar moiety.

As ureaplasmas require a complex growth medium (section 2.2) and it is known that serum components adsorb to the organism (section 1.2.1) then it is possible that this contaminating glycoprotein originates from the growth medium and indeed, a glycoprotein from the medium aligns with this contaminating protein as shown by SDS-PAGE and Concanavalin A staining (Fig 3.7, lane C).

Fig 3.5: Inhibition of metabolism of *U. urealyticum*, serotype 8 by anti-96 kDa Mabs UU8/29, UU8/5, UU8/31 and UU8/33. The organism was diluted to give a starting titre of approximately 10^2 c.c.u. ml^{-1} and incubated in the presence or absence of antibody. Samples were taken for pH measurements and for estimation of urease activity ($\mu\text{M NH}_4$).

●, pH in the absence of antibody; ○ urease activity in the absence of antibody;
▲, pH in the presence of 4 anti-96 kDa Mabs, representing the 4 epitope groups,
all at a concentration of 1/100; △ urease activity in the presence of 4 Mabs; ■ pH
in the presence of 1/100 homologous polyclonal anti-serum; □ urease activity
in the presence of 1/100 homologous polyclonal anti-serum.

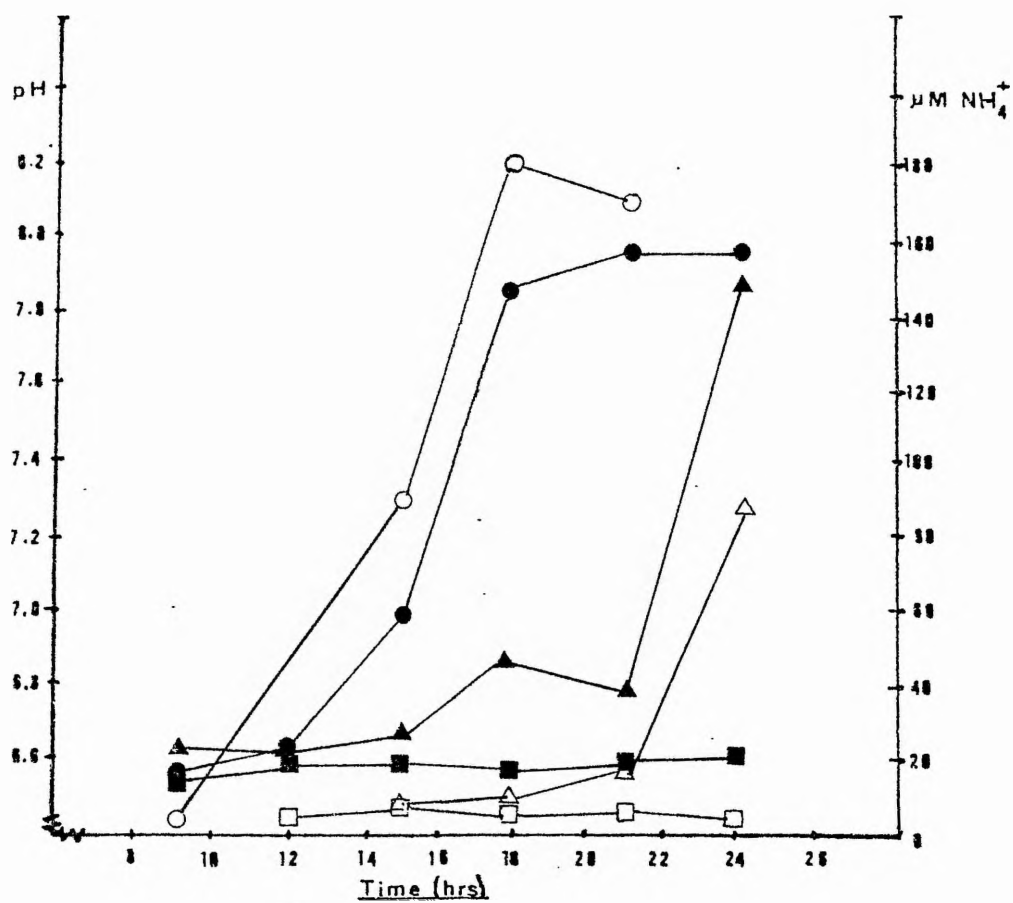


Fig 3.6: Electrophoretic analysis on a 15 % (w/v) SDS-polyacrylamide gel of the purification of the 96 kDa polypeptide visualised with Coomassie blue stain.

Lane A - *U. urealyticum*, serotype 8 (100 µg protein).

Lane B - Column flow through.

Lane C - PBS-N wash from the column.

Lane D - Partially purified 96 kDa antigen, eluted with 0.1 M glycine, pH 2.8.

Lane E - Molecular mass standards.

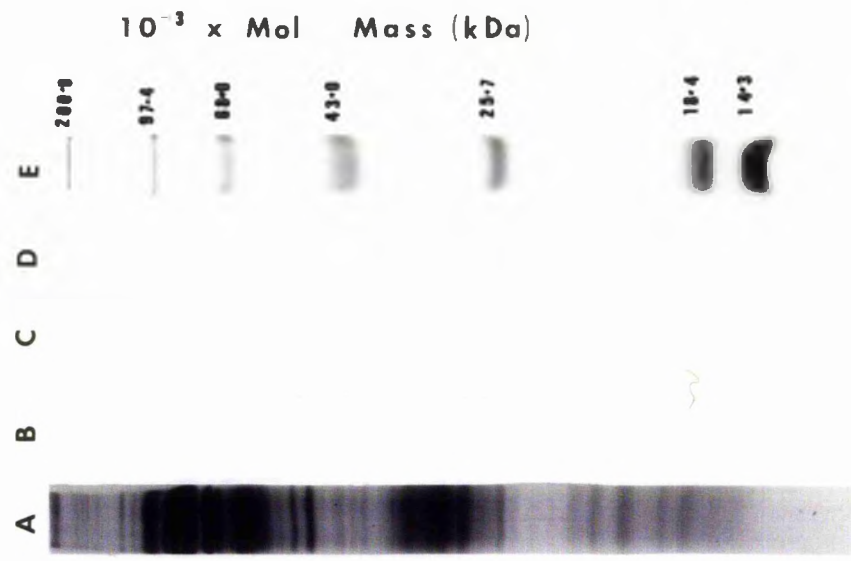
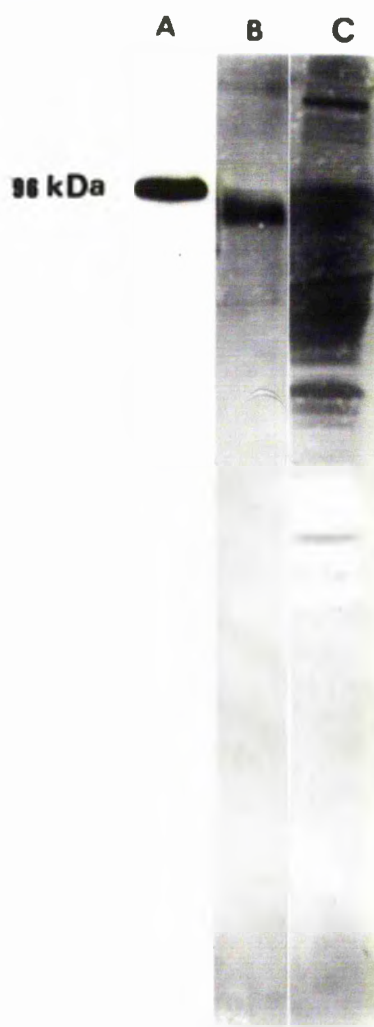


Fig 3.7: Electrophoretic separation on a 15 % (w/v) SDS-polyacrylamide gel and subjected to:-

Lane A - Immunoblot of partially purified 96 kDa polypeptide from serotype 8, probed with anti-96 kDa Mab (UU8/29) and ^{125}I -labelled Protein A.

Lane B - Partially purified 96 kDa polypeptide transferred to nitrocellulose and stained with peroxidase-linked Concanavalin A.

Lane C - Growth medium (1/10 dilution), transferred to nitrocellulose and stained with peroxidase-linked Concanavalin A.



3.2.4. SEROTYPE 8 SPECIFICITY OF THE 96 kDa ANTIGEN.

The fourteen known serotypes of *U. urealyticum* were subjected to SDS-PAGE and immunoblotting, and were probed with anti-96 kDa Mabs representing each of the four epitope groups. As shown in Fig 3.8, these Mabs appear to be serotype 8-specific and this is the first definite finding of a serotype-specific antigen in any of the fourteen serotypes of *U. urealyticum*.

3.3. THE 16 kDa AND 17 kDa ANTIGENS OF *UREAPLASMA UREALYTICUM*

Two other Mabs, designated UU8/39-40, were raised against serotype 8, one of which was of immunoglobulin class G (UU8/39). SDS-PAGE and immunoblotting showed that both Mabs recognised two polypeptides of molecular weights 16 kDa and 17 kDa (Fig 3.9) and both of these polypeptides were also recognised by homologous polyclonal serum (Fig 3.9, lane C).

3.3.1. INHIBITION OF METABOLISM.

Metabolic inhibition studies were performed, as described, using both UU8/39 and UU8/40, which recognise the 16/17 kDa polypeptides of serotype 8 and similar results were seen with both antibodies. Fig 3.10 represents one such study with Mab UU8/39 and this shows that no significant metabolic inhibitory activity was detected, suggesting that these polypeptides are not important for *in vitro* growth, and/ or that binding of the antigen by antibody has no effect on the function of these polypeptides. Again, whereas homologous polyclonal antiserum used as a positive control inhibited growth completely, a Mab raised against viral antigens and used as a negative control, had no effect.

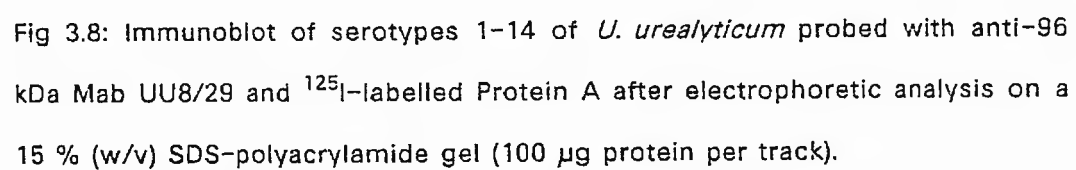
The immunoblot image is mostly blank, with only a few faint, dark spots visible. These spots are likely the result of the immunoblotting process, where specific proteins from the serotypes are detected by the anti-96 kDa Mab UU8/29 and ¹²⁵I-labelled Protein A. The spots are scattered across the page, with a notable cluster of faint spots in the upper right quadrant and a few isolated spots elsewhere.

Fig 3.8: Immunoblot of serotypes 1-14 of *U. urealyticum* probed with anti-96 kDa Mab UU8/29 and ¹²⁵I-labelled Protein A after electrophoretic analysis on a 15 % (w/v) SDS-polyacrylamide gel (100 µg protein per track).

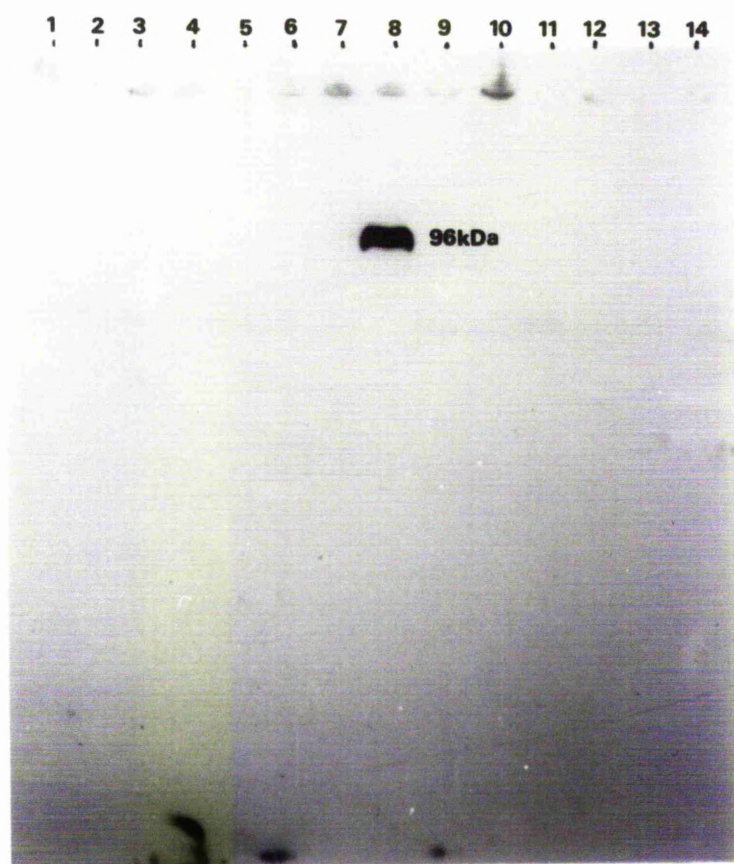


Fig 3.9: Immunoblot of *U. urealyticum*, serotype 8, probed with:-

Lane A - Anti-16/17 kDa Mab UU8/39.

Lane B - Anti-16/17 kDa Mab UU8/40 and goat anti-mouse immunoglobulins.

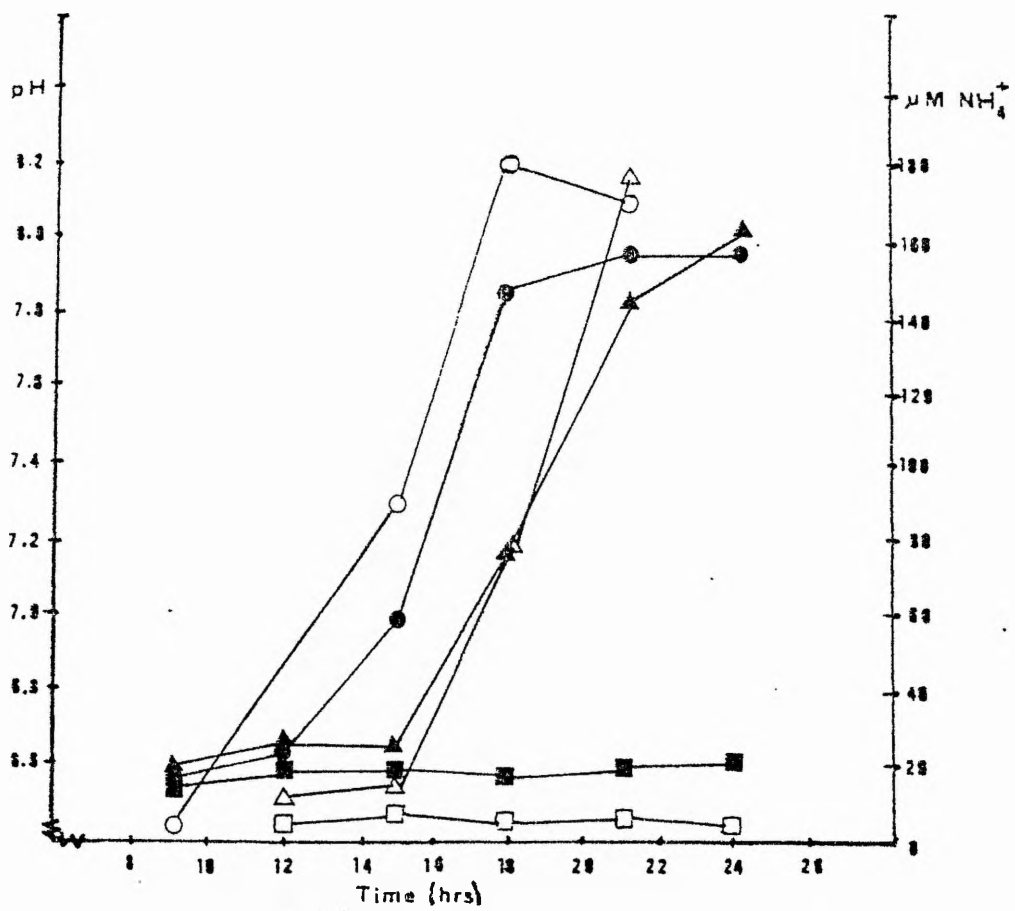
Lane C - Homologous polyclonal rabbit anti-serum.

All tracks were then probed with ^{125}I -labelled Protein A after electrophoretic separation on a 15 (w/v) % SDS-polyacrylamide gel (approximately 100 μg protein per track).



Fig 3.10: Metabolic inhibition studies using anti-16/17 kDa Mab UU8/39. The organisms were diluted to give a starting titre of approximately 10^2 c.c.u. ml^{-1} and incubated in the presence or absence of antibody. Samples were taken for pH measurements and for the estimation of urease activity ($\mu\text{M NH}_4$).

●, pH in the absence of antibody; ○, urease activity in the absence of antibody; ,
 ▲pH in the presence of 1/50 Mab UU8/39; △, urease activity in the presence of
 1/50 Mab UU8/39; ■ pH in the presence of 1/100 homologous polyclonal rabbit
 anti-serum; □ urease activity in the presence of 1/100 homologous polyclonal
 rabbit anti-serum.



3.3.2. PURIFICATION OF THE 16 kDa AND 17 kDa POLYPEPTIDES.

Attempts to purify these 16 kDa and 17 kDa polypeptides by immuno-affinity chromatography using Mab UU8/39 proved to be largely unsuccessful. A sonicated cell extract was prepared in PBS-N as described in section 2.10 and this was passed through the column which was then washed and eluted as described (section 2.10). SDS-PAGE, Coomassie blue staining and immunoblotting did not detect the presence of the 16/17 kDa polypeptides in the eluates, nor could the presence of these polypeptides be detected in the original solubilised extract (Fig 3.11, lane B). The unsolubilised cell pellet was subjected to SDS-PAGE and immunoblotting and the polypeptides were found to be associated with this pelleted material (Fig 3.11, lane A). The 16 kDa and 17 kDa antigens were eventually solubilised in a buffer containing 0.1 % (w/v) SDS and 5mM EDTA (Buffer A) (section 2.10.3). However, after elution from the column, only very small quantities of these polypeptides were detected by immunoblotting (Fig 3.11, lane D) when compared to the quantity observed in the extract prepared in Buffer A, prior to passage through the column (Fig 3.11, lane C). This may suggest that these polypeptides were remaining on the column or becoming degraded during the purification process, albeit that an attempt was made to prevent this by the addition of PMSF in buffer A, a general protease inhibitor.

Unfortunately, attempts to improve the yield of the 16/17 kDa antigen failed. SDS-PAGE, followed by Coomassie blue staining showed that other proteins co-eluted with these antigens, irrespective of how thoroughly the immuno-affinity column was washed with buffer A prior to elution (Fig 3.11, lane E). The 16/17 kDa antigens may be very closely associated with these other proteins and the use of denaturing agents may be required to break such associations. However, since the use of denaturing agents may also inactivate the immuno-affinity column, this approach was not attempted. After transfer of the eluted material to nitrocellulose sheet, and staining with peroxidase-labelled concanavalin A, no evidence of glycosylation of the 16/17

Fig 3.11: Electrophoretic analysis on a 15 % (w/v) SDS-polyacrlamide gel and immunoblotting analysis of the purification of the 16 kDa and 17 kDa polypeptides from *U. urealyticum*, serotype 8.

Lane A - Unsolubilised cell pellet prepared in PBS-N, subjected to SDS-PAGE and immunoblotting, probed with anti-16/17 kDa Mab UU8/39 and ¹²⁵I-labelled Protein A.

Lane B - Solubilised cell extract, prepared in PBS-N, probed as in Lane A.

Lane C - Solubilised cell extract, prepared in 10 mM Tris, pH 7.2, 5 mM EDTA, 0.5 % (v/v) NP40, 0.65 M NaCl, 0.1 % (w/v) NaN₃, 1 mM PMSF and 0.1 % (w/v) SDS, probed as in Lane A.

Lane D - Solubilised cell extract, as prepared in C, passed through the affinity column and eluted with 0.1 M glycine, pH 2.8 and probed as in Lane A.

Lane E - SDS-PAGE and Coomassie blue staining of the eluted extract as described in D.



kDa polypeptides was seen. Whilst this may suggest that the 16/17 kDa antigens are not glycosylated with glucose or mannose, the negative result could also be due to the low levels of material probed and/or the relative insensitivity of the test.

3.3.3. THE 16 kDa AND 17 kDa POLYPEPTIDES HAVE EPITOPES WHICH ARE SEROCLUSTER-SPECIFIC.

SDS-PAGE and immunoblotting with the anti-16 kDa/17 kDa Mab UU8/39 was carried out on all fourteen serotypes and the resultant autoradiogram is shown in Fig 3.12. UU8/39 recognises both a 16 kDa and a 17 kDa polypeptides in those serotypes belonging to the larger serocluster (Group A). However, the 17 kDa polypeptide only is recognised in the serotypes of the smaller serocluster (Group B), implying that the 16 kDa polypeptide antigen is specific to those serotypes of the larger serocluster. Using this probe, serotype 13 whose serocluster designation is undecided, falls into the larger serocluster. Thus this Mab is a useful probe for the differentiation of the two seroclusters of this organism.

3.4. 96/16/17/ kDa POLYPEPTIDES ARE MEMBRANE ANTIGENS.

Phase partitioning with Triton X-114 (section 2.13) was carried out on serotypes 8 and 1, being representatives of the two designated seroclusters of *U. urealyticum*. Fig 3.13 (a) shows both the detergent (lane B) and aqueous phases (lane C) of serotype 8 after phase partitioning, SDS-PAGE and silver staining. Many of the proteins of *U. urealyticum* serotype 8, partitioned exclusively into the hydrophilic aqueous phase (lane C), indicating the inability of these components to interact with detergent micelles. In contrast the TX-114 phase (lane B) contained approximately eleven identifiable polypeptides, representing only a small proportion of the total polypeptides of this organism (lane A). Some proteins partitioned exclusively into the detergent phase, since no similar proteins corresponding in size were observed in the aqueous phase, thus identifying a set of intrinsic proteins with hydrophobic domains capable of

Fig 3.12: Immunoblot of serotypes 1-14 of *U. urealyticum* probed with the anti-16/17 kDa Mab UU8/39 and ^{125}I -labelled Protein A after electrophoretic separation on a 15 (w/v) % SDS-polyacrylamide gel (approximately 100 μg protein per track).

1	3	6	14	13	2	4	5	7	11	8	12	9	10

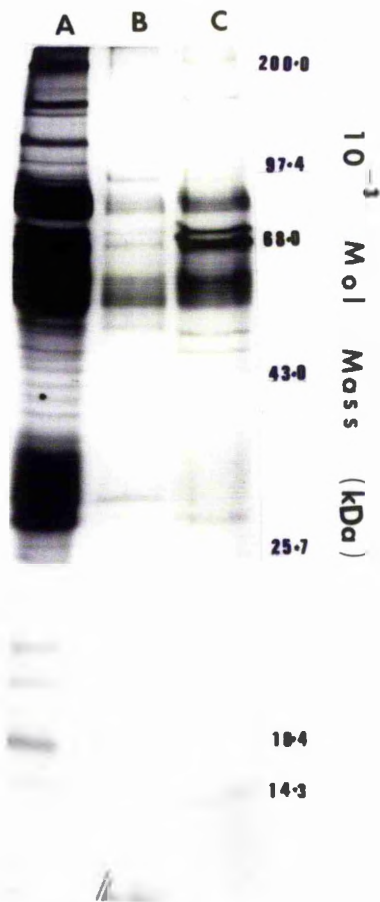


Fig 3.13 (a): Electropherogram of a 15 % (w/v) SDS-polyacrylamide gel of the polypeptides of *U. urealyticum*, serotype 8, visualised with silver stain.

Lane A - *U. urealyticum*, serotype 8, before phase partitioning with Triton X-114 (50 µg protein).

Lane B - Phase partitioning of serotype 8 with TX-114, detergent phase.

Lane C - Phase partitioning of serotype 8 with TX-114, aqueous phase.



forming micelles during isolation. Many of the proteins in the detergent phase aligned with similarly migrating proteins in the aqueous phase, these components may represent distinct, co-migrating proteins with different partitioning characteristics, or single proteins, with amphipathic properties resulting in the distribution into both phases.

Immunological identification of several of these proteins by immunoblotting demonstrated their selective properties. Serotypes 8 and 1, after phase partitioning, were subjected to SDS-PAGE and immunoblotting with an anti-96 kDa Mab (UU8/29) (Fig 3.13 (b)) (lanes A-C) and an anti-16/17 kDa Mab (UU8/39) (lanes D-G). After overnight exposure of the autoradiogram, the 96 kDa polypeptide was found exclusively in the detergent phase, (lane A) suggesting this polypeptide has major hydrophobic characteristics and is associated with the membrane. After prolonged exposure of the autoradiogram (3 days), a small proportion of the 96 kDa polypeptide was seen to partition into the aqueous phase (lane C), suggesting that it has some minor hydrophilic characteristics.

The 16/17 kDa polypeptides of serotype 8 phased entirely into the detergent phase even after prolonged exposure (lane D), with only the 17 kDa polypeptide being seen in the detergent phase of serotype 1 (lane F). This suggested that these polypeptides are also hydrophobic and most probably membrane associated.

Whole cells of serotype 8 were labelled with Bolton and Hunter reagent (section 2.14) (Fig 3.14, lane A) to identify integral membrane proteins with regions exposed to this surface-labelling reagent. SDS-PAGE analysis and autoradiography showed a discrete number of proteins accessible to surface iodination. The selectivity of labelling was established by a comparison with the far more complex pattern obtained after labelling sonicated disrupted organisms (Fig 3.14, lane H). Immunoprecipitations (section 2.15), using labelled whole cells, were carried out with an anti-96 kDa Mab (UU8/29) (lane B) and with an anti-16/17 kDa Mab (UU8/39) (lane C). These three

Fig 3.13 (b): Immunoblots of *U. urealyticum*, serotype 8, (Lanes A-E) and serotype 1 (Lanes F-G) after phase partitioning with Triton X-114 and 15 % (w/v) SDS-PAGE.

Lanes A-C - Probed with an anti-96 kDa Mab (UU8/29) and 125 I-labelled protein A.

Lanes D-G - Probed with an anti-16/17 kDa Mab (UU/39) and 125 I-labelled protein A.

Lanes A,D,F - Detergent phases.

Lanes B,C,E,G - Aqueous phases.

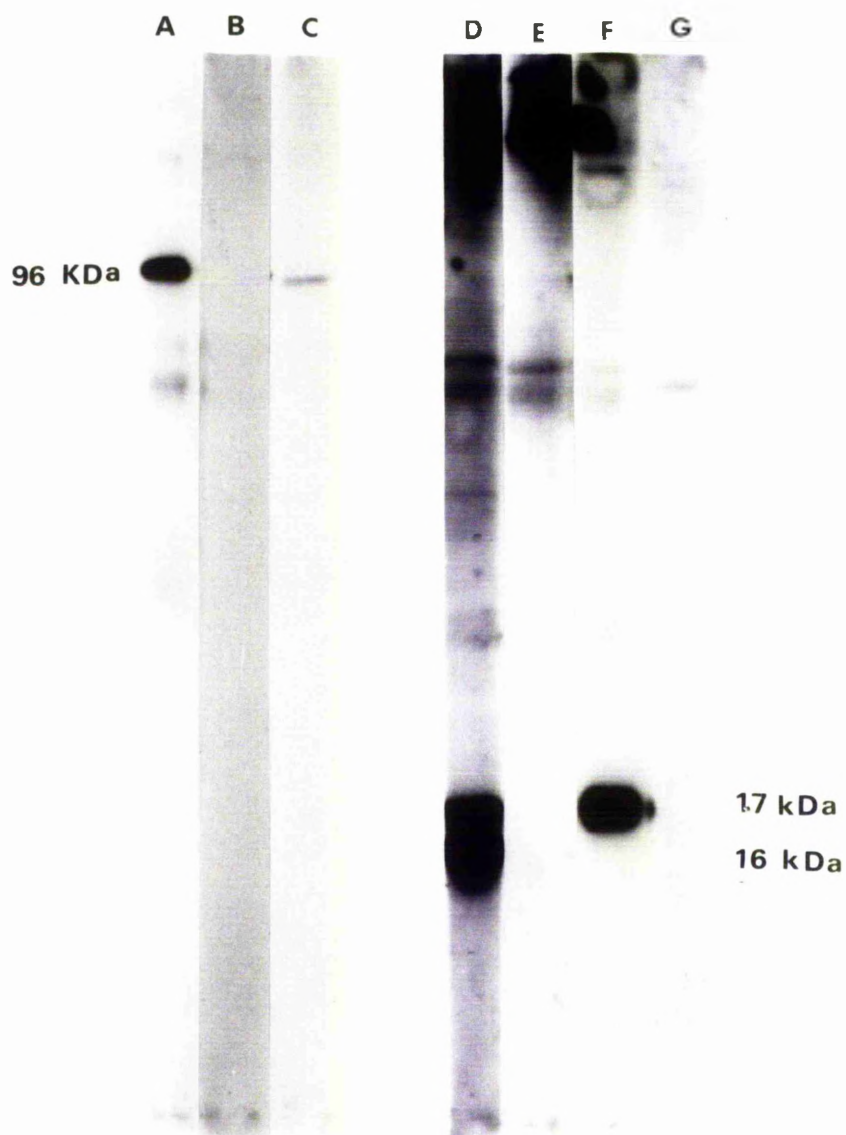


Fig 3.14: Autoradiogram of *U. urealyticum*, serotype 8 after 15 % (w/v) SDS-PAGE, showing:-

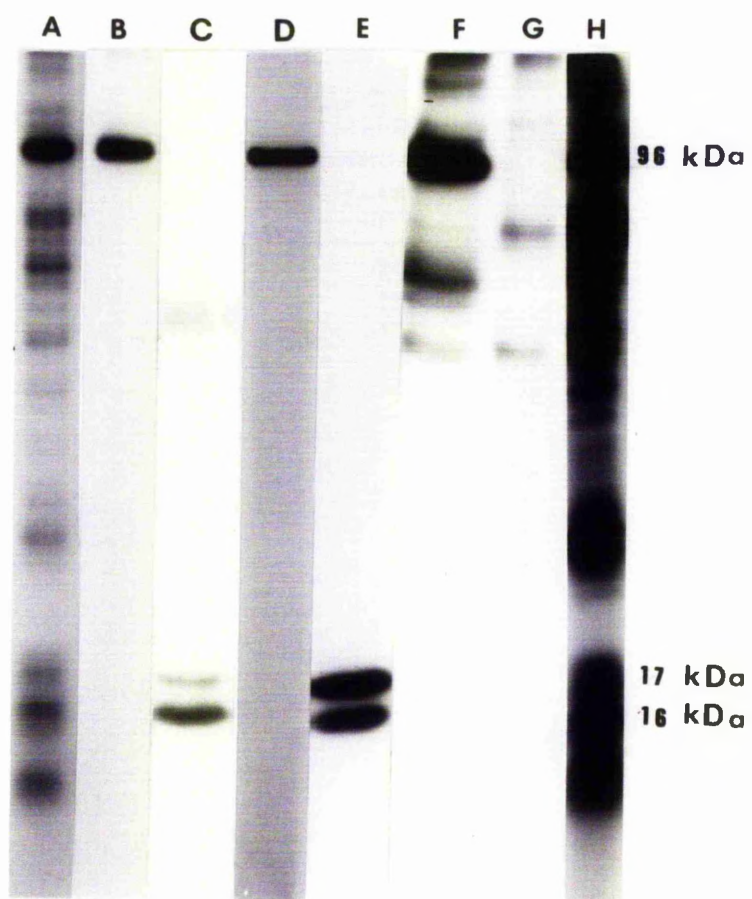
Lane A - ^{125}I Bolton and Hunter-labelled whole cells.

Lanes B-C - Immune-precipitations of soluble extract of ^{125}I -labelled whole cells of serotype 8 with an anti-96 kDa Mab (UU8/29) (B) and an anti-16/17 kDa Mab (UU8/39) (C).

Lanes D-E - Immune-precipitations of soluble extract of ^{125}I -labelled sonicated cell extract of serotype 8 with an anti-96 kDa Mab (UU8/29) (D) and an anti-16/17 kDa Mab (UU8/39) (E).

Lanes F-G - Immune-precipitations of soluble extract of ^{125}I -labelled whole cell extract of serotype 8, with homologous polyclonal anti-serum (F) and with *M. ovipneumoniae* polyclonal anti-serum (G).

Lane H - ^{125}I Bolton and Hunter-labelled sonicated cells of serotype 8.



polypeptides were all labelled and the results suggest that these are surface expressed antigens. The 96 kDa polypeptide appears to be labelled more strongly than the 16/17 kDa polypeptides. This could have been due to two reasons: (1), the Bolton and Hunter reagent labels lysine groups, thus the more available lysine groups within the polypeptide the better the labelling, or (2), the 96 kDa polypeptide is a major antigen of serotype 8 and thus will appear to be more strongly labelled due to the large amount of this polypeptide available. It is also possible that the 16/17 kDa polypeptides are not surfaced expressed and that the contents of a few lysed cells were labelled. However, the cells were washed extensively to prevent this occurring and such an explanation would not correlate with the results from the phase separation using TX-114.

Sonicated cell extract of serotype 8 (labelled with Bolton and Hunter reagent) was also subjected to immunoprecipitations as above. The 16/17 kDa polypeptides appeared to label more strongly using this method (Fig 3.14, lane E), whereas the 96 kDa polypeptide labelled to the same extent (lane D). This suggests that the 16/17 kDa polypeptides are integral membrane proteins which may be exposed on both sides of the membrane.

Homologous polyclonal antiserum and polyclonal antiserum raised against *Mycoplasma ovipneumoniae* were used as positive and negative controls respectively (lanes F and G).

3.5. UPTAKE OF ^3H -PALMITIC ACID BY *U. UREALYTICUM*

The single lipid bilayer surrounding this organism (section 1.2.1) represents the only interface through which these prokaryotes interact with the environment. Proteins associated with the membrane must participate in a variety of essential functions, including transport systems for the organisation of nutrients and/or adhesion and colonisation of host cell surfaces. Little is known about the exact structure of these

proteins that mediate their interaction with the lipid bilayer and it has yet to be determined whether common mechanisms exist by which membrane proteins are anchored or processed in these organisms.

To assess the possibility that some intrinsic membrane proteins of *U. urealyticum* might be associated with lipids, ureaplasmas were metabolically labelled with ^3H palmitic acid (section 2.16).

Fig 3.15 (lane A) shows that approximately 26 discrete polypeptides labelled with ^3H palmitic acid and of these, approximately 11 polypeptides (apparent molecular masses of 126, 96, 63, 55, 50, 42, 39, 33, 31, 24 and 16 kDa) were intensely labelled. A large number of labelled polypeptides were also detected by silver staining after SDS-PAGE (lane B) and since several of these appeared to align with the labelled polypeptides in lane A, this suggests the presence of several lipid-modified proteins in this organism.

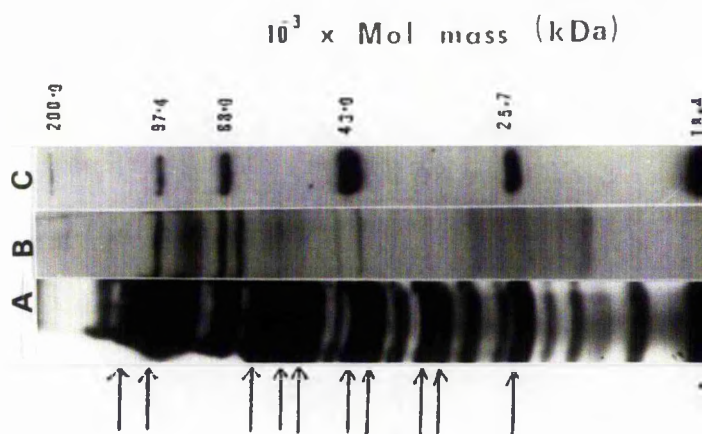
The ^3H palmitic acid-labelled organisms were subjected to TX-114 phase separation and the phases analysed by SDS-PAGE and autoradiography. Fig 3.16 shows the resultant detergent phase (lane A) and the resultant aqueous phase (lane B). Approximately 20 labelled bands could be visualised in the detergent phase which is close to the number of bands observed in the labelled whole organism (Fig 3.15, lane A). Those fatty acid-labelled components present in low quantity are lost by this technique. As the labelled organisms were not subjected to chloroform-methanol extraction as described by Bricker *et al.* (1988), several of the labelled bands may represent noncovalently bound lipid which would account for the few bands seen in the aqueous phase. Although the majority of the ^3H palmitic acid labelled-96 kDa antigen (as shown by immunoprecipitations) was shown to be in the detergent phase, a significant but smaller proportion was seen in the aqueous phase. This confirms the results found previously by phase partitioning (Fig 3.13, lanes A-C) and confirms the amphipathic nature of this antigen. It should be pointed out however, that the

Fig 3.15: Electrophoretic analysis on a 15 % (w/v) SDS-polyacrylamide gel of ^3H palmitic acid-labelled *U. urealyticum*, serotype 8.

Lane A - Autoradiogram of ^3H palmitic acid-labelled serotype 8.

Lane B - Silver stain of ^3H palmitic acid-labelled serotype 8.

Lane C - Molecular mass standards.



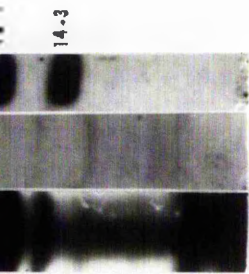


Fig 3.16: Autoradiogram of ^3H palmitic acid labelled *U. urealyticum*, serotype 8 after electrophoresis on a 15 % (w/v) SDS-polyacrylamide gel.

Lanes A,B - ^3H palmitic acid-labelled serotype 8 subjected to phase separation with Triton X-114.

Lane A - Detergent phase.

Lane B - Aqueous phase.

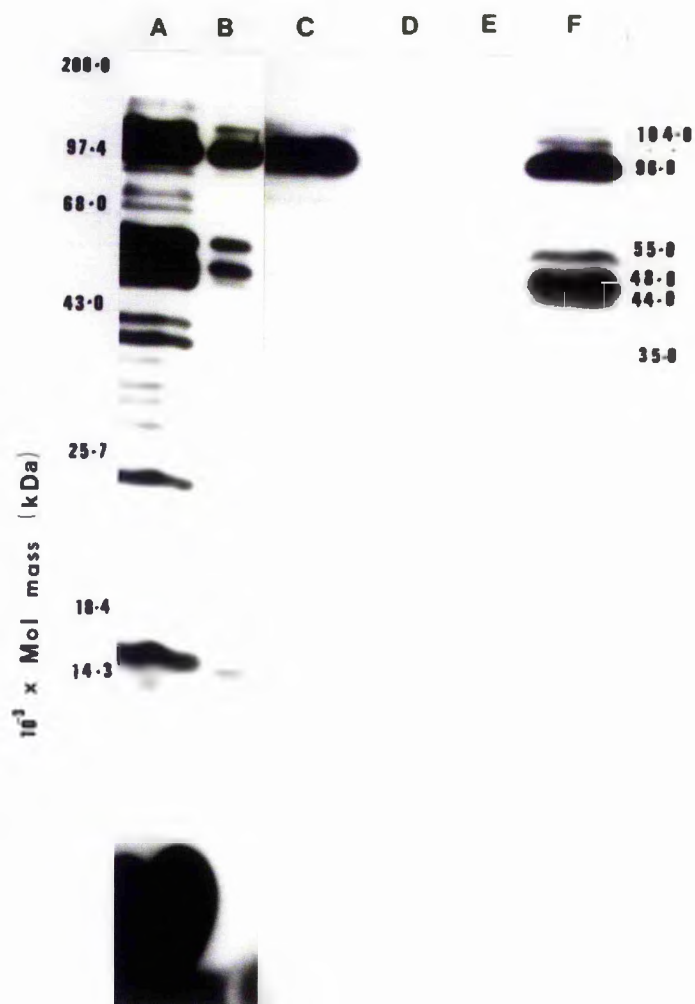
Lanes C-F - Immunoprecipitations of ^3H Palmitic acid-labelled serotype 8 with:-

Lane C - An anti-96 kDa Mab UU8/29.

Lane D - An anti-16/17 kDa Mab UU8/39.

Lane E - An anti-urease Mab UU8/17.

Lane F - Homologous polyclonal antiserum.



proportion of this antigen seen in the aqueous phase may be mis-leading since this autoradiogram was exposed for 3 months. Of the 5 bands seen in the aqueous phase, one of which represents the 96 kDa antigen (as discussed above) it is possible that all 5 components, have amphipathic properties resulting in their distribution into both phases.

3.5.1. IDENTIFICATION OF INTEGRAL MEMBRANE PROTEINS OF *U. UREALYTICUM* BY MABS AND HOMOLOGOUS POLYCLONAL ANTISERUM.

Immunoprecipitations of ^3H -palmitate-labelled proteins were carried out using the anti-96; anti-16/17 kDa Mabs (UU8/29, UU8/39 respectively), the anti-urease Mab (UU8/17), homologous polyclonal antiserum and polyclonal antiserum raised against *M. ovipneumoniae*. The resultant autoradiogram is shown in Fig 3.16. It can be seen that several ^3H -palmitic acid labelled proteins were precipitated by the homologous polyclonal serum (lane F) and that the 96 kDa major surface membrane protein was also labelled with this lipid (Fig 3.16, lane C) and appears to be the most intensively fatty-acid- labelled component.

Neither the 16/17 kDa nor urease antigens were precipitated, (lanes D and E respectively) suggesting these proteins are not modified with palmitic acid. Thus the 16 kDa polypeptide observed in Fig 3.15 (lane A) is not apparently the 16 kDa polypeptide recognised by Mab UU8/39.

The functional significance of covalent fatty acid attachment is not known, but Schmidt *et al.*, (1979) have proposed that bound fatty acids may act to anchor the protein in the lipid bilayer and thus form a structural role. Cholesterol has been proposed to provide added strength to the membranes of these organisms (Rottem, 1979). It has also been proposed (Dahl *et al.*, 1983) that proteolipid may provide an alternative device for maintaining membrane and thus cellular integrity.

CHAPTER 4
THE UREASE ENZYME OF *UREAPLASMA UREALYTICUM*

4.1. THE UREASE ENZYME.

Ureaplasma species differ from all the other mycoplasmas due to the possession of a urease enzyme which hydrolyses urea to carbon dioxide and ammonia. Urease activity has been detected in a large number of bacteria, but the ureaplasmas are the only organisms known to depend on urea for growth (see section 1.4). Ureaplasma urease appears to be a major constituent of the cytoplasm and has been shown to have a considerably higher specific activity than jack bean urease (Precious *et al.*, 1987). This urease enzyme is also thought to play a role in the pathogenicity of the organisms (section 1.6).

Despite the apparent importance of the ureaplasma urease, knowledge of the structure and properties of the enzyme is fragmentary. Early attempts to characterise the enzyme in crude cell extracts as well as more recent efforts directed at the purification of the enzyme, have been problematic due to the extremely low yields of ureaplasmas and the high level of medium contamination of the harvested organisms.

The production of an anti-urease Mab (UU8/1) enabled this enzyme to be purified by immuno-affinity chromatography as described by Precious *et al.* (1987). This purified enzyme was then used as an immunogen to produce a further 18 anti-urease Mabs (section 2.4(a)) (discussed later, 4.7).

4.2. SUBUNIT STRUCTURE OF UREAPLASMA UREASE.

Using anti-urease Mab (UU8/1) or anti-urease Mab (UU8/17) (see 4.7), active purified urease was prepared by immuno-affinity chromatography, followed by dialysis against PBS at 4°C (section 2.11). Using the assay method of Bradford (1976) (section 2.5(a)) it was shown that approximately 1 mg of enzyme was recovered from the cell pellet obtained from a 10 litre culture. SDS-PAGE of freshly purified enzyme, prepared in 50 % (v/v) glycerol, without prior boiling (section 2.6.1(i)), revealed two closely

migrating protein bands when stained with either Coomassie blue stain or with the more sensitive silver stain (Fig 4.1, lane A). Both bands were shown to be enzymically active with the Fishbein stain or with the lead acetate stain (Fig 4.1, lanes B and C) although the band of higher molecular mass was the more intense both by staining and in enzymic activity. As the enzyme was not denatured prior to electrophoresis, accurate determination of molecular masses was not possible, but both bands were within the 170–200 kDa range. To calculate the molecular mass of the enzyme more accurately, FPLC (section 2.18) was carried out on freshly prepared urease. A single major protein peak, shown by the Berthelot reaction to be the active urease, was eluted and when subjected to non-denaturing SDS-PAGE was shown to contain only the two enzymically active protein bands. By comparison of the elution volume of this major peak with the elution volumes of the molecular mass standards, the molecular mass of the single peak corresponding with urease activity was shown to be 190 kDa.

After boiling of the freshly prepared enzyme in both the presence or absence of denaturing agents, the samples were subjected to SDS-PAGE. All enzymic activity had been lost (Fig 4.1, F) and total degradation to three subunits with apparent molecular masses of 72 ± 4 kDa, 14 ± 2 kDa and 11 ± 1 kDa (Fig 4.1, lane E) had occurred. However, it is noteworthy that the two smaller subunits could only be seen after electrophoresis on a 15 % (w/v) polyacrylamide gel. When the boiled enzyme was electrophoresed on a 10 % (w/v) polyacrylamide gel, these smaller subunits merged with the dye front (Fig 4.1, lane I). After SDS-PAGE of boiled urease prepared in 'denature mix' (2.6.1(ii)) had been carried out, the gels were stained with coomassie blue and subjected to densitometry (section 2.19). The individual peak areas were calculated and after taking the apparent molecular mass of the subunits into consideration, it was shown that they were present in equimolar ratios (72 kDa:14 kDa:11 kDa::1:0.99:0.95), consistent with the native enzyme being a hexamer ($72 \text{ kDa}_2:14 \text{ kDa}_2:11 \text{ kDa}_2$). Similar patterns were obtained with all batches of the purified enzyme.

Fig 4.1: Electrophoretic analysis of polypeptides associated with purified urease.

Lane A - 10 % (w/v) gel; freshly prepared urease, coomassie stain.

Lane B - 10 % (w/v) gel; freshly prepared urease, Fishbein stain.

Lane C - 10 % (w/v) gel; freshly prepared urease, Lead acetate stain.

Lane D - 15 % (w/v) gel; molecular mass standards.

Lane E - 15 % (w/v) gel; urease after boiling (2 min), coomassie stain.

Lane F - 15 % (w/v) gel; urease after boiling (2 min), Fishbein stain.

Lane G - 15 % (w/v) gel; thawed enzyme; coomassie stain.

Lane H - 15 % (w/v) gel; freshly prepared urease, coomassie stain.

Lane I - 10 % (w/v) gel; urease after boiling (2 min), coomassie stain.

Lane J - 10 % (w/v) gel; Molecular mass standards.

(approximately 20 μ g protein per track).

A B C



D E F G H I J

200.0 200.0

97.4 97.4

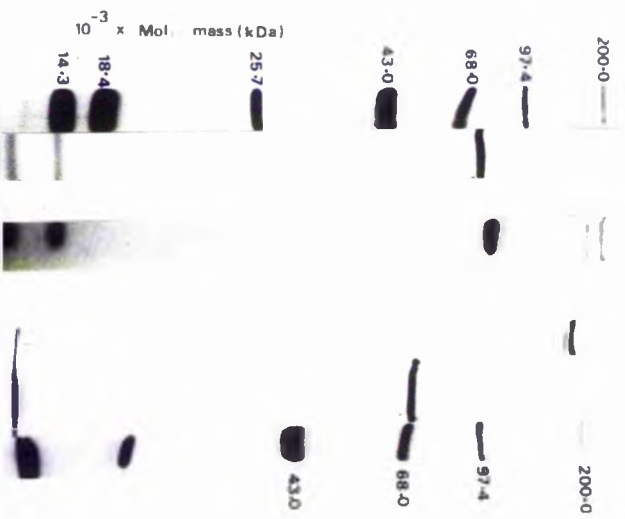
68.0 68.0

43.0 68.0

25.7 43.0

10^{-3} x Mol. mass (kDa)

18.4
14.3



4.3. STABILITY OF THE ENZYME.

Storage of the purified urease at -70°C with or without added glycerol, in siliconised or non-siliconised tubes, followed by thawing to 20°C , resulted in a partial breakdown to the three subunits, and a 50–55 % loss of activity as shown using the Berthelot reaction. The active protein bands and the subunits were visualised after non-denaturing SDS-PAGE with coomassie stain (Fig 4.1, lane G) and no additional components were detected by silver-staining. Work in the laboratory by Frost also showed that at 20°C , the addition of urea, B-mercaptoethanol or of "denaturing mix" in the absence of boiling did not appear to accelerate enzyme breakdown over a period of 20 mins (Thirkell *et al.*, 1989).

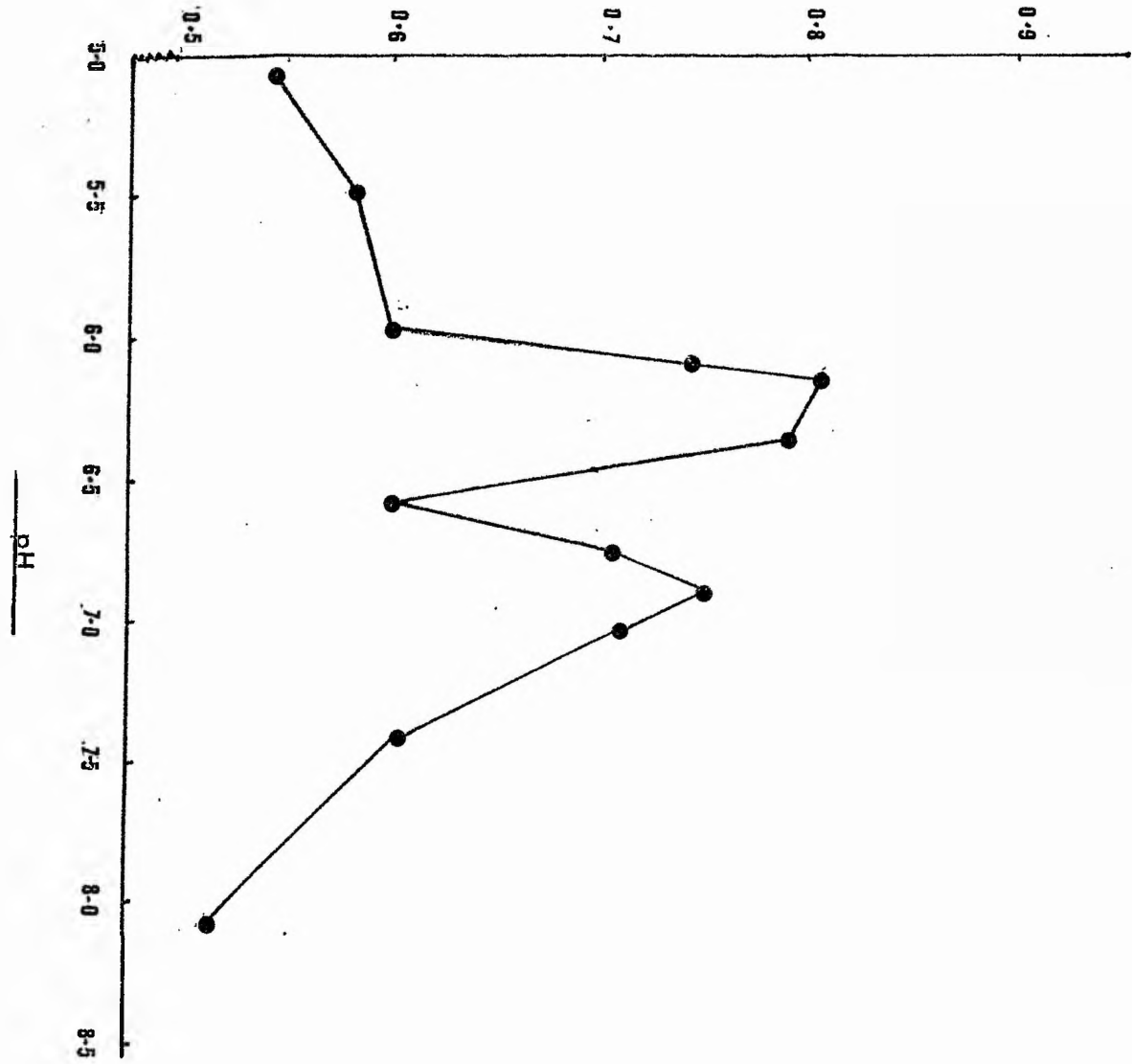
4.4. pH OPTIMA FOR ENZYME ACTIVITY.

As discussed in section 2.20, the pH activity of this enzyme was evaluated over a wide range of pH (pH 2.2–10.7) by Frost (Thirkell *et al.*, 1989). No enzyme activity was detected below pH 4.3 or above pH 8.5 and the optimum pH for activity was shown to be pH 6.9 (Thirkell *et al.*, 1989). To verify the actual pH optimum of the enzyme a narrower pH range of pH 5.0–8.1 was used as described in section 2.20. The results obtained are shown in Fig 4.2. Two pH optima are apparent at pH 6.15 and pH 6.9 with more activity at pH 6.15.

Previously reported pH optima for urease from *U. urealyticum* serotype 8, have been at pH 5–6 (Masover *et al.*, 1976), pH 7.2–7.5 (Eng *et al.*, 1986) and also pH 7.5 (Blanchard *et al.*, 1988). However, Swanberg *et al.* (1978) also detected the presence of two pH optima at pH 5.0 and pH 7.0 with reduced enzymic activity at pH 6.0 and these workers suggested that the two optima may be due to the presence of different isoenzymes which were reported by Delisle (1977). The differences in pH optima may be due to the different buffer systems used, but the overall pattern reported by Swanberg is similar to the results reported here.

Fig 4.2: pH activity curve for purified urease, using 0.1 M citrate buffer.

A-680 nm



4.5. THE UREASES OF *U. UREALYTICUM*, SEROTYPES 1-14.

U. urealyticum, serotypes 1-14 were subjected to non-denaturing PAGE (section 2.6.2) and to denaturing SDS-PAGE (section 2.6.1). In both cases, the urease activity in the gels after electrophoresis was detected with lead acetate (section 2.17). In the latter case, the SDS was removed from the gel prior to staining by thorough washing with PBS-N since SDS has an inhibitory effect on most enzymic activity (Lacks *et al*, 1979). On addition of urea, the urease metabolises this to ammonia which causes a localised increase in pH and leads to the dissociation of the lead acetate to form soluble ammonium acetate and an insoluble white lead precipitate in the area of pH increase as seen in Figs 4.3 (a) and (b). After electrophoresis using the 'native' gel system, a single active urease band was seen in all 14 serotypes (Fig 4.3 (a)). After SDS-PAGE, two active bands are apparent (Fig 4.3 (b)) in the 170-190 kDa range and it is possible that the lower molecular weight band with less enzymic activity may represent a slightly denatured though still active form of the urease.

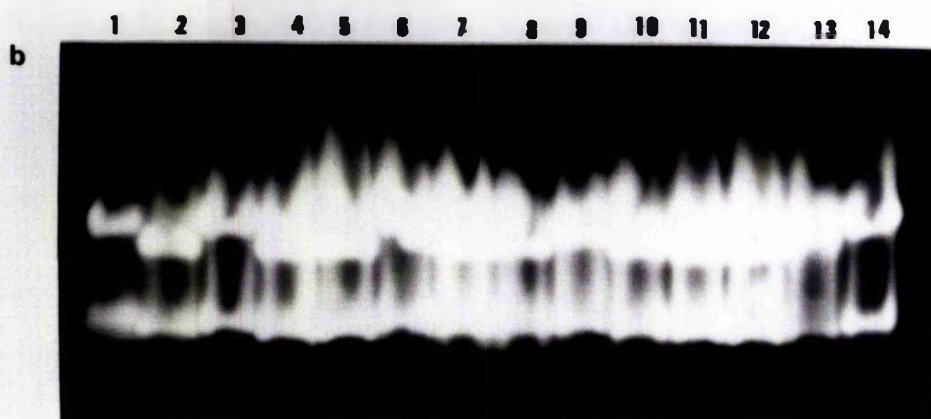
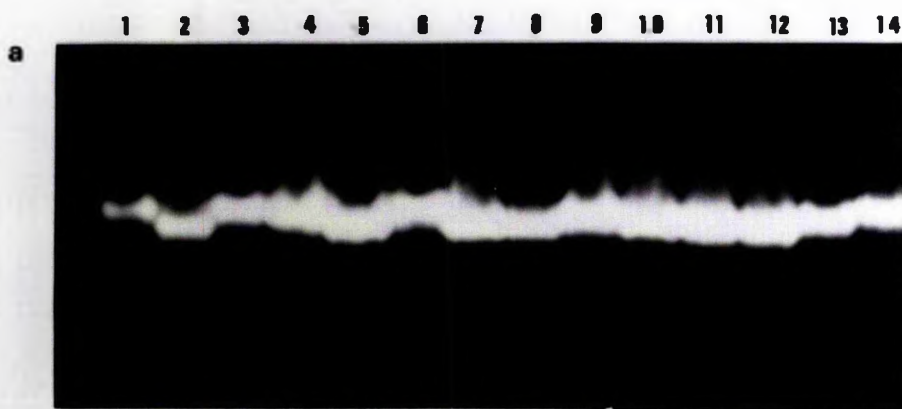
The molecular masses of the urease of the 14 serotypes appear to differ irrespective of the gel system used and such differences may reflect minor variations in amino-acid sequence, in structure and/or organisation of the enzyme. The ureases of the serotypes belonging to the small serocluster (serotypes 1,3,6 and 14) behaves similarly and appear to have a slightly higher molecular mass, those of serotypes 2,5,7,8,10,11 and 12, appear to have the lowest molecular mass and those of serotypes 4,9 and 13 appear to have a molecular mass which is between the masses of the above. These findings of molecular mass variations for the urease of the different serotypes of *U. urealyticum* is consistent with previously reported data (Davis *et al*, 1988).

Fig 4.3: *U. urealyticum*, serotypes 1-14 subjected to:-

(a) Non-denaturing (7.5 % w/v) polyacrylamide gel electrophoresis.

(b) Denaturing (7.5 % w/v) SDS-PAGE.

Stained with lead acetate (100 µg protein per track).



4.6. UREASE CATCH ASSAY.

This assay was initially reported by Precious *et al.* (1987) but was subsequently found to be unreliable with results appearing to depend upon the make of 96-well plate used and to vary from day-day. After trying various modifications to the assay system, it was found that the desired reliability and reproducibility of the assay, irrespective of the make of 96-well plate used, could be achieved if the wells of the 96-well plates were pre-coated with goat anti-mouse (GAM) immunoglobulins as described in section 2.21. A typical assay result is shown in Fig 4.4 where anti-urease Mabs (UU8/1-lane 3, UU8/17-lane 10, UU8/11-lane 11) and anti-96 kDa Mabs (UU8/29-34-lanes 4-9) as negative controls, were used in the pre-coated wells, and where doubling dilutions of a log culture of *U. urealyticum*, serotype 8 (1/10-1/320 dilutions in rows A-F) provided the urease antigen. As expected, blue colour developed only in those pre-coated wells to which anti-urease Mabs had been added (lanes 3, 10 and 11).

4.7. ANTI-UREASE MABS.

Although one such Mab (UU8/1) was available from a previous fusion (Precious *et al.*, 1987), the use of immunoaffinity-purified urease from *U. urealyticum*, serotype 8 as immunogen, allowed 18 further anti-urease Mabs (designated UU8/11-UU8/28) to be raised. Using the radioimmune assay technique, 5 of these (UU8/12-13, UU8/17-18, UU8/26) were shown to be of immunoglobulin class G.

All 19 Mabs recognised the 'native' enzyme of serotype 8 in the catch test without affecting enzymic activity suggesting that the epitope(s) recognised by these Mabs was/were not on, or presumably near to, the active site of the enzyme.

Although the autoradiogram is over exposed, homologous polyclonal serum was seen to recognise both active bands of the purified urease after 'non-denaturing'

Fig 4.4: Urease catch assay with doubling dilutions (1/10 - 1/320) of a logarithmic culture of *U. urealyticum*, serotype 8 (10^7 c.c.u. ml⁻¹) (rows A-F).

The plate was pre-coated with a 1/200 dilution of GAM in PBS.

Lane 1 - Blank (no antigen added).

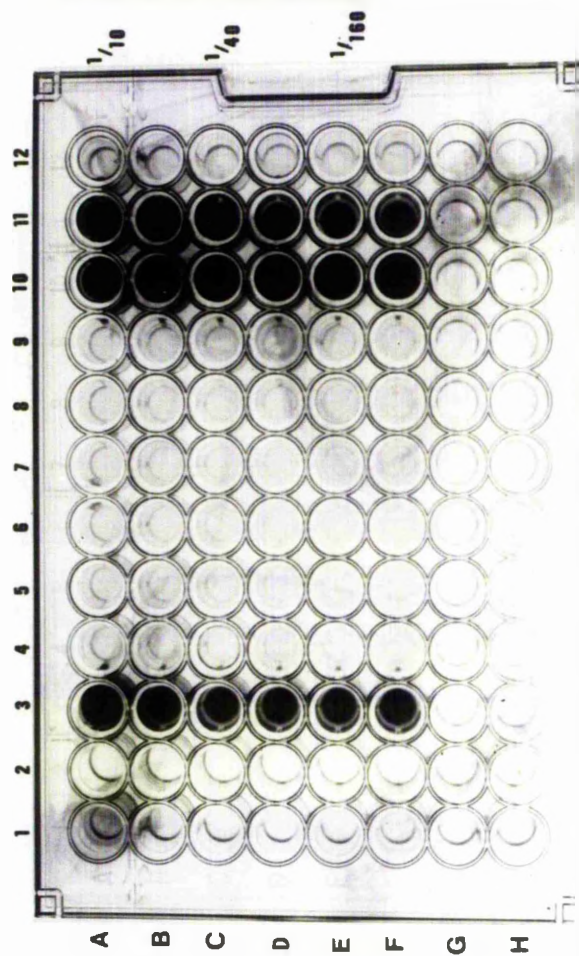
Lane 3 - Wells coated with anti-urease Mab UU8/1.

Lanes 4-9 Wells coated with anti-96 kDa Mabs UU8/29-34.

Lane 10 - Wells coated with anti-urease Mab UU8/10.

Lane 11 - Wells coated with anti-urease Mab UU8/11.

Dilution of U. urealyticum



PAGE and immunoblotting (Fig 4.5, lane B), as did all 18 anti-urease Mabs. Typical results with two of these Mabs are shown in Fig 4.5 (lanes D and F).

After SDS-PAGE and immunoblotting with denatured enzyme, it was found that the 72 kDa sub-unit was recognised by homologous polyclonal serum (Fig 4.5, lane A) and reproducibly by only 6 of the 18 Mabs (UU8/12-13, UU8/17-18, UU8/26-27). Two such examples are seen in Fig 4.5 (lanes C and E). The two smaller sub-units of the urease (14 kDa and 11 kDa) were not recognised on immunoblot by homologous polyclonal serum or by any of the 19 anti-urease Mabs.

4.8. CROSS-REACTIONS OF MABS WITH THE UREASE OF THE OTHER 13 SEROTYPES OF *U. UREALYTICUM*

It was subsequently shown (see section 4.4) that the 6 Mabs which had been shown by SDS-PAGE and immunoblotting to recognise the 72 kDa sub-unit of the urease of serotype 8, between them recognised three different epitopes on the enzyme. A representative Mab from each of these different epitope groups (UU8/12, UU8/17, UU8/26) was then used to probe the other 13 serotypes of the organism.

After PAGE and immunoblotting, all these Mabs were shown to recognise both active bands of the native ureases (data not shown). However, after SDS-PAGE and immunoblotting of denatured ureases from the serotypes, UU8/17 was seen to recognise the 72 kDa sub-unit of the ureases from only 11 of the 14 serotypes. This sub-unit was not recognised in serotypes 3, 6 and 14 (Fig 4.6). Although these three serotypes belong to the smaller serocluster of *U. urealyticum*, the 72 kDa sub-unit of the urease from the other serotype of that smaller cluster (serotype 1), was recognised. After SDS-PAGE and immunoblotting of denatured ureases, the 72 kDa sub-unit was detected in all 14 serotypes by the other two Mabs used (UU8/12, UU8/26) (data not shown).

Fig 4.5: Immunoblot of fresh and boiled urease of *U. urealyticum*, serotype 8.

Lanes A,C,E - Urease after boiling (2 min).

Lanes B,D,F - Freshly prepared urease.

Lanes A,B - Probed with homologous polyclonal serum (1/800 dilution in 50:50 (v/v) PBS-N growth medium).

Lanes C,D - Probed with anti-urease Mab (UU8/17) belonging to epitope group A (1/500 dilution as above).

Lanes E,F - Probed with anti-urease Mab (UU8/13) belonging to epitope group B (1/500 dilution as above).

All tracks were probed with ^{125}I -labelled Protein A after electrophoresis on a 15 % (w/v) SDS-polyacrylamide gel ($\sim 20 \mu\text{g}$ protein per track).

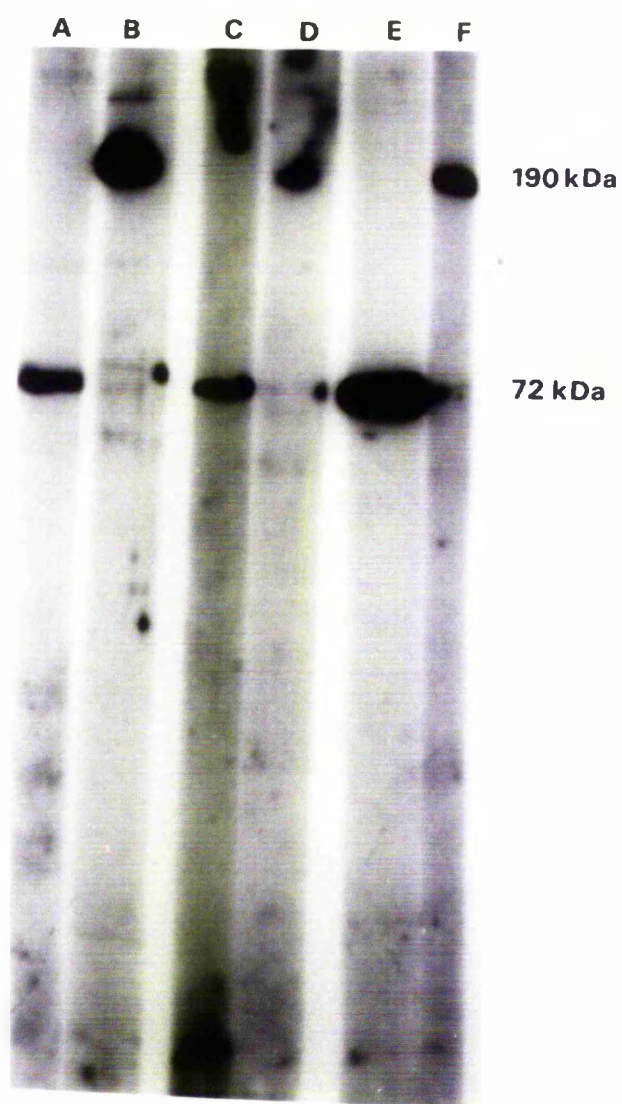
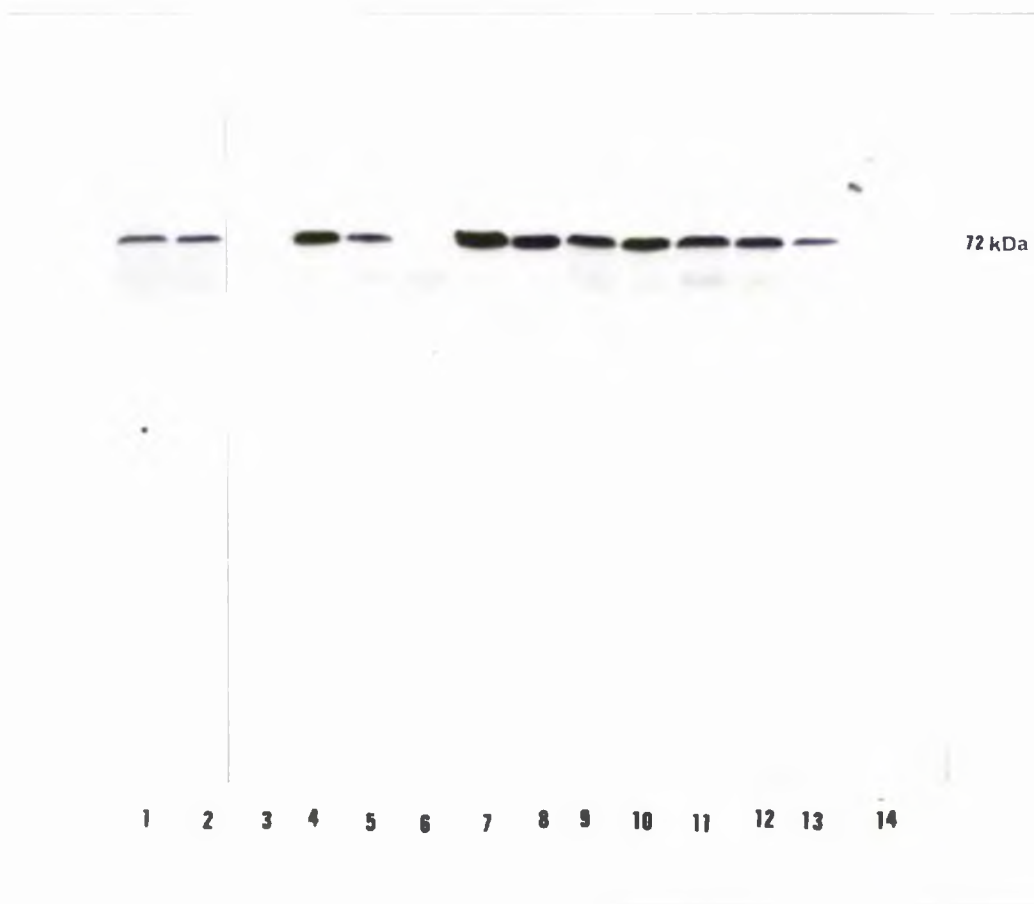


Fig 4.6: Immunoblot of serotypes 1-14 of *U. urealyticum* probed with the anti-urease Mab (UU8/17) and ^{125}I -labelled Protein A after electrophoretic separation by 15 % (w/v) SDS-PAGE (~ 100 μg protein per track).



4.9. COMPETITION ASSAYS FOR DETERMINATION OF EPITOPES ON THE UREASE.

As described in section 2.8, these assays were carried out using two different techniques which both gave similar results. It was shown that the 19 available anti-urease Mabs, between them recognised at least 4 distinct epitopes on the enzyme. Using the catch assay system, a typical result is shown in Fig 4.7. The wells of both 96-well plates were pre-coated with GAM Ig and then coated with anti-urease Mab UU8/1. On plate 1, competition assays were carried out against UU8/1 (lane 3), and against UU8/11-UU8/19 (lanes 4-12). On plate 2, competition assays were carried out against UU8/20-UU8/28 (lanes 3-11). On plate 1, competition to varying degrees is evident in lanes 3 (UU8/1), 4 (UU8/11), 7 (UU8/14), 10 (UU8/17) and 11 (UU8/18) and on plate 2, in lanes 3-6 (UU8/20-UU8/23). This suggests that these 9 Mabs all recognise the same epitope on the enzyme. Assays of this type were repeated three times in each case, initially coating the wells with a Mab which had shown no competition in previous assays. The groupings of Mabs recognising each of four distinct epitopes were:-

Group A: UU8/1, UU8/11, UU8/14, UU8/17-18, UU8/20-23

Group B: UU8/12-13

Group C: UU8/24-28

Group D: UU8/15-16, UU8/19.

The binding of the urease by two Mabs during these assays may have inhibited the activity of the enzyme, which in turn, would have given misleading results such that inhibition would be mistaken for competition. To ensure that this was not the case, competition assays were carried out with Mabs from each of the four epitope groups as follows:-

(a) the urease enzyme was incubated with both Mabs simultaneously in the wells

Fig 4.7: Urease competition assay using the enzyme catch test.

Plates 1 and 2 pre-coated with GAM (1/200 in PBS) and Mab UU8/1 (1/100 in PBS).

Lane 1 – Blank (no antigen added).

Well 2 A – Positive control (no competing antibody added).

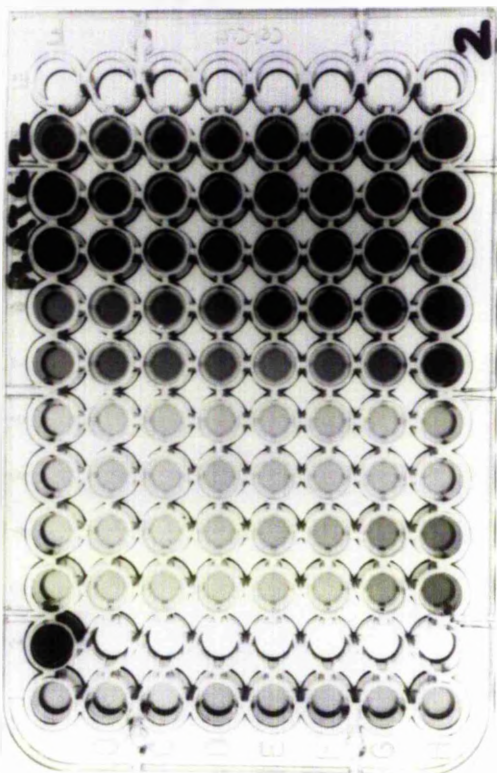
Plate 1:

Lanes 3–12 – Competition respectively with UU8/1, UU8/11–UU8/19 (2-fold dilutions from 1/50 (A) to 1/6400 (H)).

Plate 2:

Lanes 3–11 – Competition respectively with UU8/20–UU8/28 (2-fold dilutions from 1/50 (A) to 1/6400 (H)).

PLATE 2



of a 96-well plate (Fig 4.8, side A), and

(b) competitions assays were carried out as normal, i.e. the urease was incubated with one antibody prior to incubation in the wells of the plate with the second Mab (Fig 4.8, side B).

As shown in Fig 4.8 competition is seen only on side B of the plate (lanes 7 and 8) suggesting that incubation of the urease simultaneously with the two Mabs did not inhibit the activity of the enzyme. As expected competition is only seen where both Mabs belong to the same epitope group.

To confirm these results, the second competition assay method using purified radiolabelled Mabs (section 2.8) was carried out. A typical result representing Mabs from the four epitope groups is shown in Fig 4.9, where the anti-urease Mab UU8/17 was radiolabelled. Using UU8/17 competition is evident in Row A (homologous unlabelled antibody) and rows C,D,E and F (corresponding to Mabs UU8/11,UU8/14, UU8/20, UU8/22). No competition is evident in rows B,G,H,I,J and K (corresponding to Mabs UU8/1, UU8/12, UU8/25-26, UU8/15 and UU8/19). With the exception of Mab UU8/1 these results confirm those observed using the urease catch assay method. Mab UU8/1 may or may not belong to epitope group A, however, the discrepancy seen here may be due to the higher affinity of Mab UU8/17 for the urease enzyme using this method.

One Mab from each of the four epitope groups was used in the catch assay to probe various bacteria which were known to possess a urease enzyme (section 2.1) but no cross reactions were observed. Thus, the Mabs appear to be specific for ureaplasma urease only.

4.10. CELLULAR LOCATION OF UREAPLASMA UREASE.

It has been reported previously that the urease is located in the cytoplasm of the

Fig 4.8: Verification of the competition assay results using the urease catch assay.

The plate was pre-coated with GAM (1/200 in PBS) and Mab UU8/17 (1/100 in PBS).

Lanes 1-6 - Urease incubated with two Mabs simultaneously.

Lanes 7-11 - Urease incubated with the second antibody prior to addition to the 96-well plate.

Lanes 1 & 7 - UU8/17 and UU8/17 (both from epitope group A).

Lanes 2 & 8 - UU8/17 and UU8/1 (both from epitope group A).

Lanes 3 & 9 - UU8/17 and UU8/13 (from epitope groups A & B respectively).

Lanes 4 & 10 - UU8/17 and UU8/26 (from epitope groups A & C respectively).

Lanes 5 & 11 - UU8/17 and UU8/16 (from epitope groups A & D respectively).

(2-fold dilutions from 1/50 (A) to 1/6400 (H)).

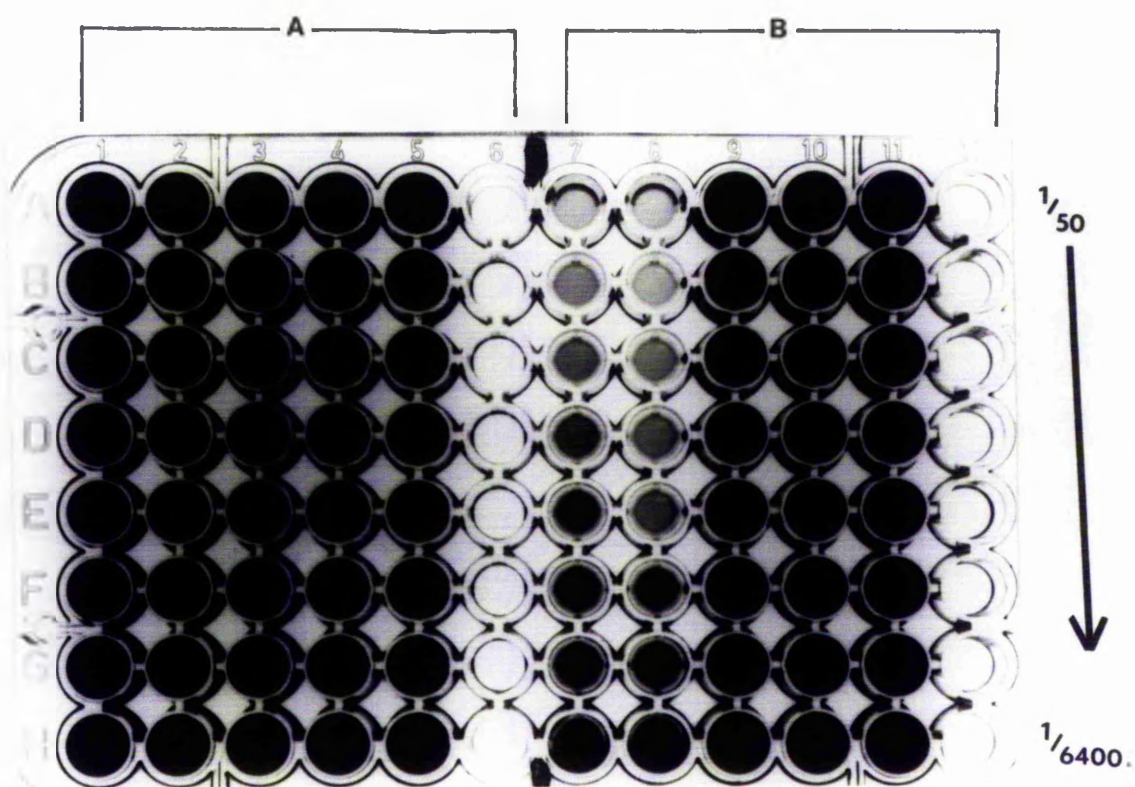


Fig 4.9: Competition assays with ^{125}I -labelled anti-urease Mab UU8/17 and other unlabelled anti-urease Mabs.

4-fold dilutions of unlabelled antibody, 1/20-1/81920.

A: Competition against homologous unlabelled antibody.

B-L: Competition against other unlabelled anti-urease Mabs.

**4 FOLD DILUTIONS OF "COLD" ANTIBODY IN
APPROPRIATE DILUTIONS OF LABELLED
ANTIBODY**



cell (Vinther, 1976; Masover *et al.*, 1977a). To confirm this, *U. urealyticum*, serotype 8, was first subjected to phase partitioning with Triton X-114 (section 2.13). The resultant detergent and aqueous phase were subjected to SDS-PAGE and immunoblotting with the anti-urease, Mab UU8/17. In Fig 4.10 (lane A (detergent phase); lane B (aqueous phase)), it is seen that the 72 kDa sub-unit of the urease partitioned exclusively into the hydrophilic aqueous phase. Subsequently, both whole cells (Fig 4.10, lane C) and sonicated cell extract (Fig 4.10, lane F) were labelled with ^{125}I using the Bolton and Hunter reagent (section 2.14) and it is obvious that many more proteins are labelled in the latter preparation. The ^{125}I -labelled whole cells and the ^{125}I -labelled sonicated cell extract were then subjected to immunoprecipitations with the anti-urease Mab UU8/17. Using this technique, none of the three urease sub-units were detected in the surface labelled whole cells (lane E), but both the 72 kDa and the 14 kDa sub-units were detected in the labelled sonicated cell extract (lane H). Again, when immunoprecipitations of these two ^{125}I -labelled preparations were carried out using homologous polyclonal serum, no urease sub-units were detected in the labelled whole cells (lane D) but both the above mentioned sub-units were again detected in the labelled sonicated cell extract (lane G).

All of these findings are consistent with the cytosolic location of the urease and this was confirmed further by the use of immunogold labelling (chapter 5).

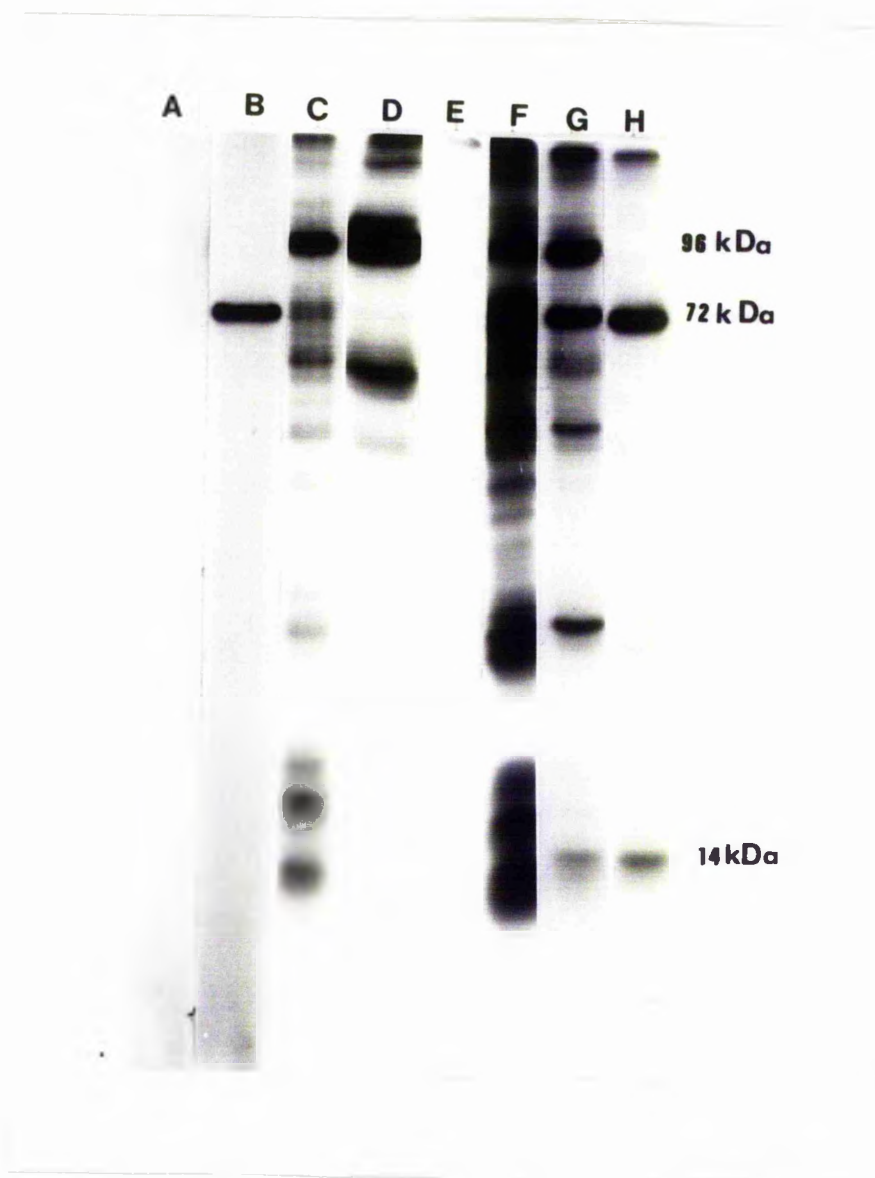
Fig 4.10: Use of immunoblotting and immunoprecipitation to establish the cellular location of the urease of *U. urealyticum*, serotype 8.

Lanes A & B - Autoradiogram of immunoblots probed with the anti-urease Mab UU8/17 of the detergent (A) and aqueous phases (B) after phase partitioning with Triton X-114 and SDS-PAGE on a 15 % (w/v) polyacrylamide gel.

Lanes C & F - Autoradiogram of ^{125}I -labelled whole cells (C) and of ^{125}I -labelled sonicated cell extract (F) after SDS-PAGE (as above).

Lanes E & H - Autoradiogram of immunoprecipitations, using Mab UU8/17 with ^{125}I -labelled whole cells (E) and with ^{125}I -labelled sonicated cell extract (H) after SDS-PAGE (as above).

Lanes D & G - Autoradiogram of immunoprecipitations, using homologous polyclonal serum, with ^{125}I -labelled whole cells (D) and with ^{125}I -labelled sonicated cell extract (G) after SDS-PAGE (as above).



CHAPTER 5
THE ULTRASTRUCTURE OF *UREAPLASMA UREALYTICUM* SEROTYPE 8.

5.1. THE ULTRASTRUCTURE OF *U. UREALYTICUM*, SEROTYPE 8.

The ultrastructure of *U. urealyticum* was examined by electron microscopy (section 2.22) using cells grown and harvested as described in section 2.2, i.e. in the presence of 20 % horse serum. Cells were prefixed before centrifugation as recommended by Maniloff *et al.* (1965), but it should be noted that Anderson and Barile (1965) did not find that prefixing *Mycoplasma hominis* cells before centrifugation had any advantage. Indeed, Whitescarver and Furness (1975) reported that centrifugation of ureaplasma cells at 14000 g for 30 minutes did not distort or lyse the cells as shown by light microscopy.

As discussed previously (section 1.2(i)), serum components from the medium are known to adsorb to the cells and this caused severe problems as can be seen in Fig 5.1 (a-d). Much of the material examined had an amorphous appearance as shown in Fig 5.1 (c), and the presence of recognisable organisms as shown in a,b and d was rare.

This problem was previously reported by Black *et al.* (1972) who replaced horse serum with PPLO fraction after the observation of an accumulation of extracellular material which obscured the electron micrographs.

In agreement with Whitescarver and Furness (1975), the cells were mostly seen as spherical or ovoid. Rod-shaped or filamentous structures as reported by Black *et al.* (1972), were not seen. The size of the majority of cells observed here ranged from 0.4 μm to 0.8 μm in diameter though occasionally smaller (0.1–0.2 μm) structures were observed. Whether these smaller structures represent viable ureaplasmas is unknown. Larger (1.4 μm) cells were also observed, these cells were mostly empty and probably represent lysed, dead cells. The diameter of the amorphous material observed was within the 0.4 μm –0.8 μm size range and may therefore represent badly fixed organisms.

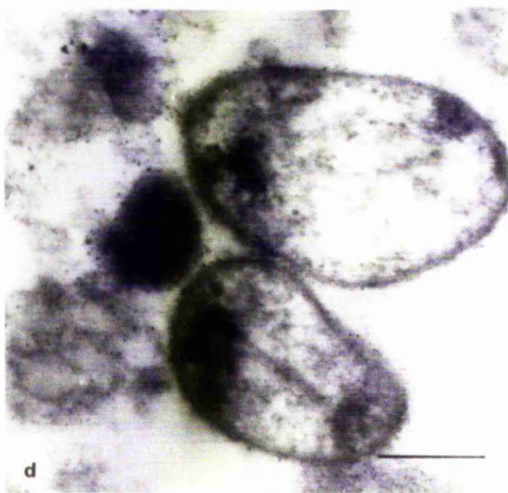
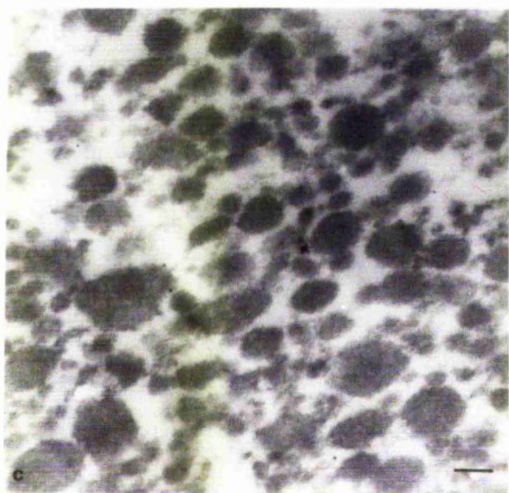
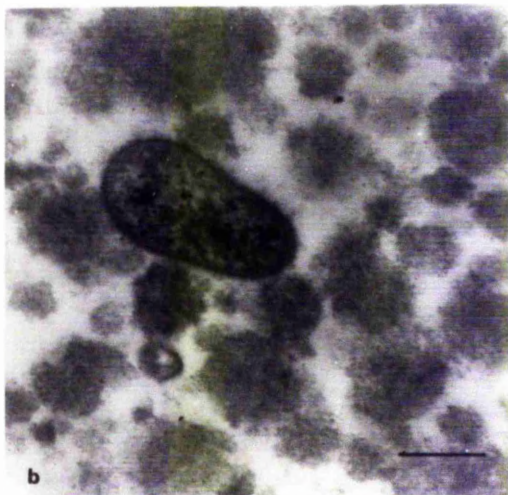
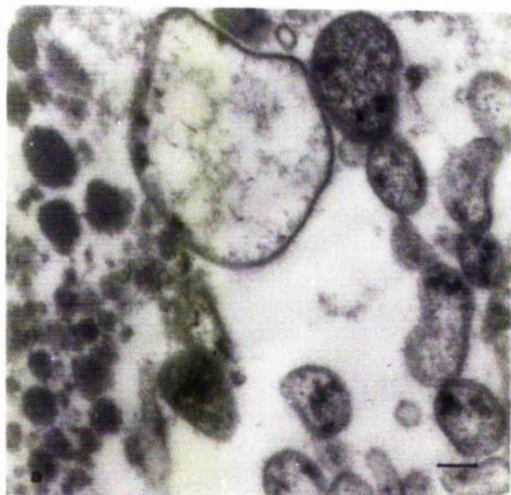
Fig 5.1: Culture of *U. urealyticum*, serotype 3 grown in a medium containing 20 % (v/v) horse serum, pre-fixed with 2.5 % (v/v) glutaraldehyde in PBS and post-fixed in 1 % (w/v) osmium tetroxide in PBS. Bar represents 0.2 μ m.

(a) Several recognisable ureaplasmas, a large organism absent of cytoplasmic material, and other amorphous structures can be seen (X 34,000).

(b) Field showing only one recognisable ureaplasma organism, surrounded by other amorphous material (X 74,000).

(c) A field showing only amorphous structures (X 34,000).

(d) Two ureaplasmas surrounded by a 'fuzzy' layer which obscures the fine structure of the membrane (X 95,000).



As reported by Black *et al.* (1972), the smaller cells (0.4 μm) were more electron dense and filled with homogenous cytoplasmic material compared to the larger cells. These authors claimed that this electron dense material represented ribosomes, either in randomly distributed or partly arranged in closely square-packed groups which were arranged to form corn-cob like patterns.

The presence of a tripled layered membrane could be seen surrounding most cells, although some organisms appeared to be covered with a 'fuzzy' layer (Fig 5.1, (d)), which obscured the fine structure of the membrane. This layer may represent the hair-like structures reported by Black *et al.* (1972) and Williams (1967) (see section 1.2.1), and/ or may be the ruthenium red-staining capsule reported by Robertson and Smook (1976). Alternatively, it may of course represent medium components adsorbed to the cell.

Black *et al.* (1972) also reported the presence of delicate filaments within the cytoplasm which were later identified as DNA but these were not seen here.

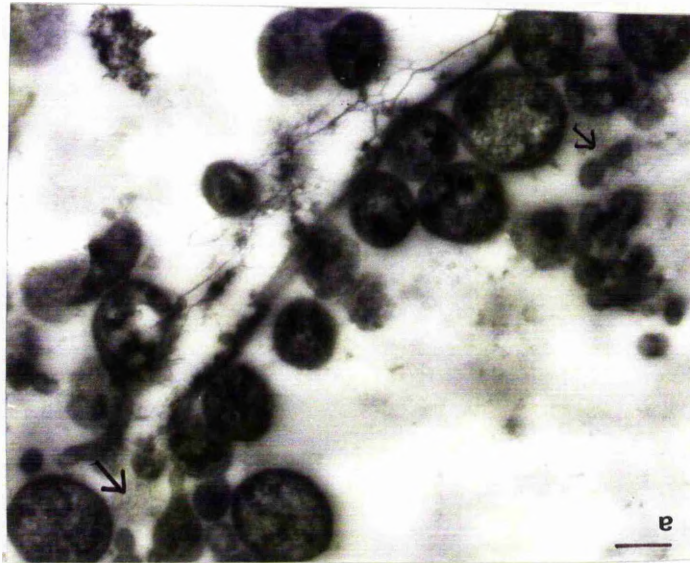
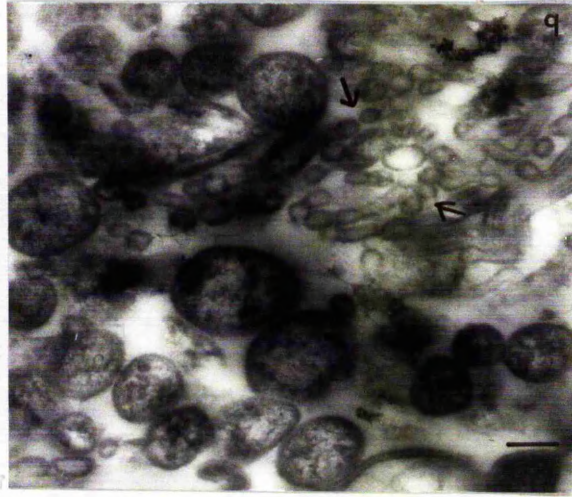
As reported previously (Precious *et al.*, 1987), growth of ureaplasmas in medium containing foetal calf serum removes most of the medium contamination. Cells were grown in this way, prefixed as before and the resultant electron micrographs can be seen in Fig 5.2. The cells observed were almost identical to those seen previously except the amorphous material was no longer present and many many more organisms could be found. Whitescarver and Furness (1975) reported the presence of budding ureaplasmas and what appears to be budding ureaplasmas can be seen in Fig 5.2(a). The presence of a 'fuzzy' layer was still observed on many cells suggesting that this layer is not composed of medium contaminants.

Occasionally, what appears to be short chains of very small (0.1 μm) ureaplasmas were seen (Fig 5.2 (b)), these may be real or they may have been artifacts.

Fig 5.2: Culture of *U. urealyticum*, serotype 8 grown in a medium containing 10 % (v/v) foetal calf serum, pre-fixed in 0.5 % (v/v) glutaraldehyde in PBS (30 min), and embedded in LR White resin. Bar represents 0.2 μ m.

(a) Large number of ureaplasmas, with densely packed cytoplasm and several of what appear to be budding ureaplasmas (arrows) (X 32,000).

(b) Ureaplasmas and chains of smaller coccoid structures (arrows) (X 28,000).



5.2. IMMUNOCYTOCHEMISTRY.

Previous work (chapters 3 and 4) has indicated that the 96 kDa, 16 kDa and 17 kDa polypeptides are integral membrane antigens of *U. urealyticum*, serotype 8, whereas the urease enzyme is cytosolic. To confirm the location of these antigens, immunocytochemistry (section 2.23) was carried out using LR White resin and Protein A gold.

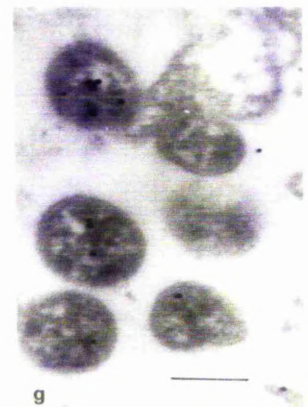
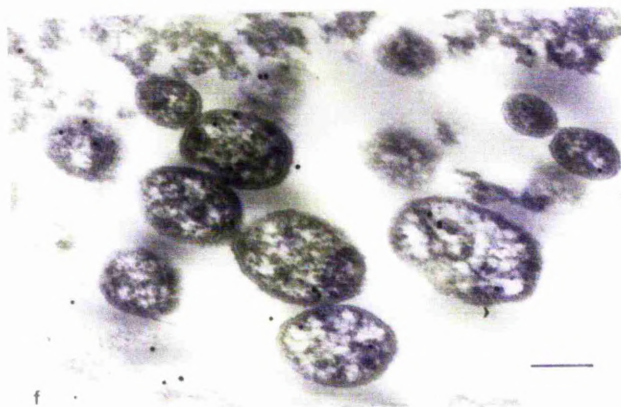
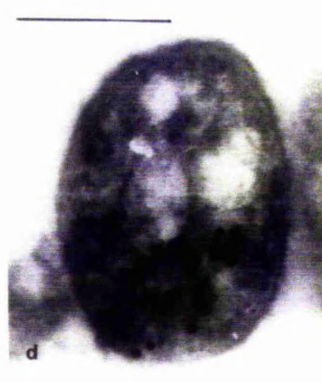
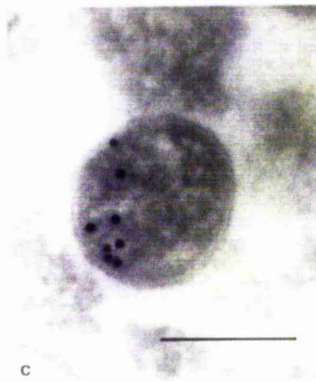
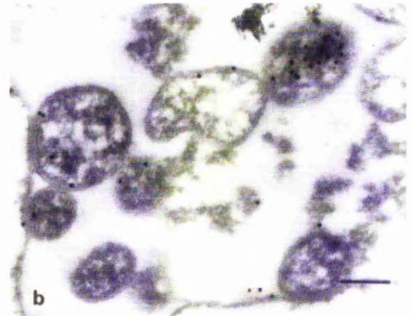
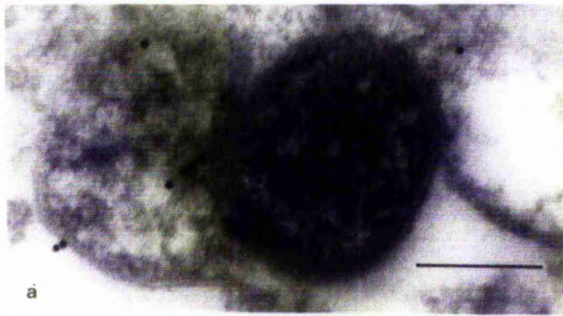
LR White resin has several advantages over traditional araldite resins which can be exploited for the localisation of antigens in sections of fixed and embedded tissue under the electron microscope. This resin is hydrophilic and allows the passage through ultrathin sections of aqueous solutions such as antibodies. Both membrane and cytosolic structures are stable within this resin, even without prior fixation with osmium which often destroys antigenicity. This resin also tolerates rapid, partial dehydration, accepting tissues from 70 % (v/v) ethanol and thus these tissues have improved antigenicity compared to those tissues which have been fully dehydrated.

Glutaraldehyde, which was used previously for the examination of the ultrastructure of this organism, was again used here as this fixative has a greater power to cross-link proteins than does formaldehyde, but as this may reduce antigenicity the concentration used and the fixation times given were very low. As fixation with osmium was avoided, the cells were more prone to distortion, thus after dehydration, the cells were placed in LR White diluted in ethanol as this prevents tissue shrinkage.

Cells were first probed with an anti-urease Mab (UU8/17) and 10 nm Protein A gold; the resultant electron micrographs can be seen in Fig 5.3. In most cases, cells were cytoplasmically labelled but on occasions, gold particles could be seen associated with other material. The smaller, more densely packed cells were more intensely labelled than the larger emptier cells. This may suggest that the urease

Fig 5.3: Electron micrographs of ultrathin sections of *U. urealyticum*, serotype 8, prepared as for Fig 5.2. Stained with an anti-urease Mab UU8/17 and Protein A gold (10 nm). Bars represent 0.2 μm .

(a) X 115,000; (b) X 49,000; (c) X 125,000; (d) X 141,000; (e) X 79,000; (f) X 58,000; (g) X 68,000.



enzyme is lost from the cell as it ages and loses integrity and this may explain why gold particles were seen outwith the cytoplasm. The presence of urease outwith the cytoplasm was also confirmed using the urease catch test with whole cells, i.e. no PBS-N was included. Negative controls, either excluding antibody or Protein A gold, were also examined and one such example can be seen in Fig 5.4 (a). Some of the cells appeared to be poorly labelled (Fig 5.3 (f)) and this could be due to several reasons, such as the antigenicity of the urease being destroyed by the fixation and/or the polymerisation methods used. In addition, the antibodies may not satisfactorily penetrate the sections unless they are ultrathin.

The 96 kDa antigen was not satisfactorily labelled using the above method as only a small proportion of the cell membrane was labelled (Fig 5.5), and this is not indicative of this polypeptide being a major membrane antigen. Thus a second method of labelling was carried out (section 2.23) which was suitable for labelling surface-expressed antigens only.

This second approach was very successful and the majority of all cell membranes were intensively labelled with gold particles (Fig 5.6). As labelling was only successful prior to fixation, this may suggest that this antigen was destroyed by the glutaraldehyde fixative or that this antigen was inaccessible to the Mab due to the cross-linking caused by the fixative. Controls were performed as for the urease labelling and an example can be seen in Fig 5.4 (b).

This method was also carried out using Mab UU8/39 which recognises the 16/17 kDa polypeptides in order to confirm that these antigens were also membrane associated. Using this method, no labelling of the cells could be seen, but the results of the Bolton and Hunter labelling had suggested these antigens may be located on both the internal and external surfaces of the membrane and results from the purification of these antigens suggested that they are intimately associated with other cellular proteins such that they are available to the antibody only after sonication or

Fig 5.4: Electron micrographs showing negative controls. Bars represent 0.2 μm .

(a) Negative control for the anti-urease Mab; *U. urealyticum*, stained with Protein A gold only (X 25,000).

(b) Negative control for the anti-96 kDa Mab; *U. urealyticum*, stained with Protein A gold only (X 43,000).

(c) Negative control for the anti-16/17 kDa Mab; *U. urealyticum*, permeabilised with PBS-N prior to staining with Protein A gold (X 50,000).

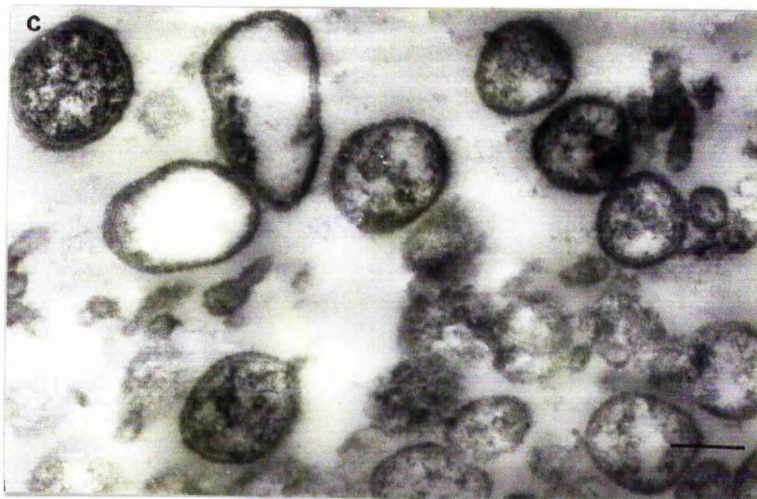
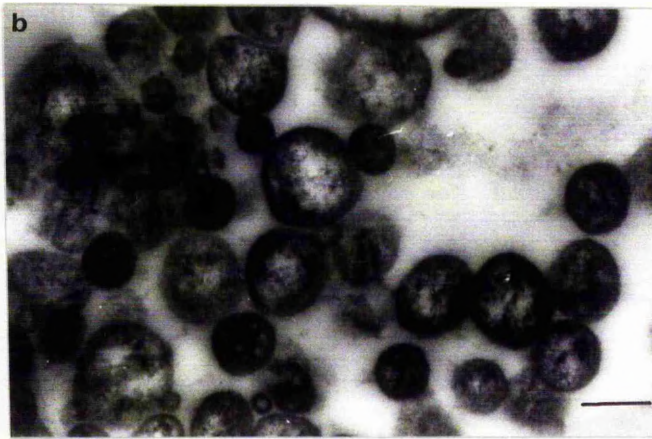
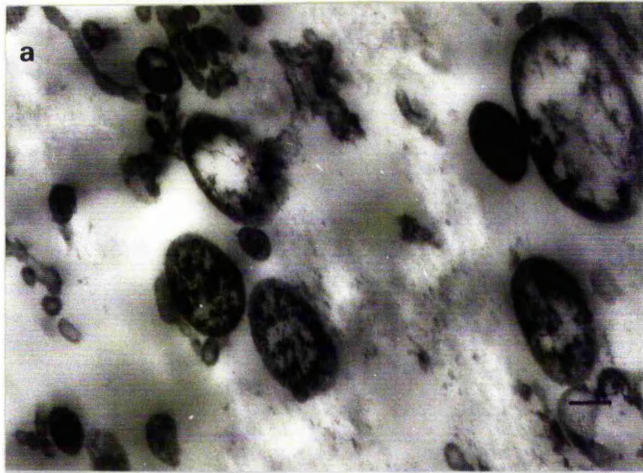


Fig 5.5: Electron micrograph of an ultrathin section of *U. urealyticum*, serotype 8, prepared as in Fig 5.2 and stained with the anti-96 kDa Mab UU8/29 and Protein A gold (X 112,000). Bar represents 0.2 μm .

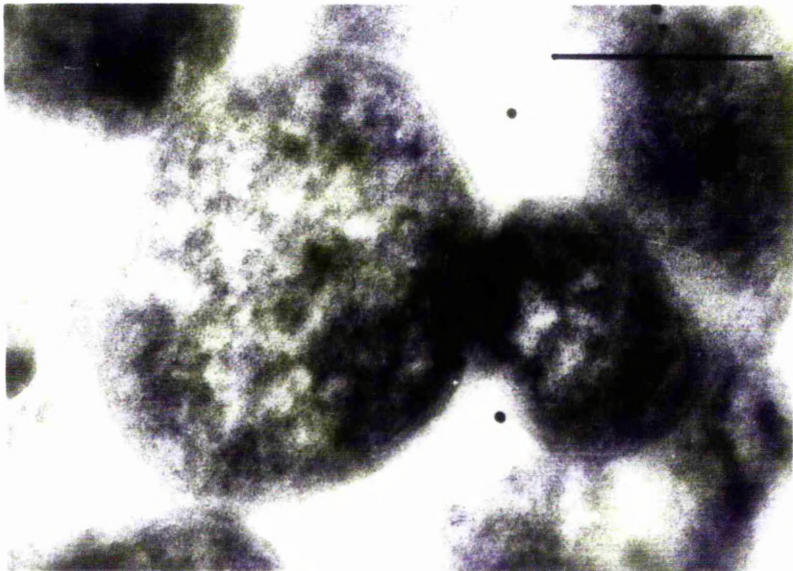
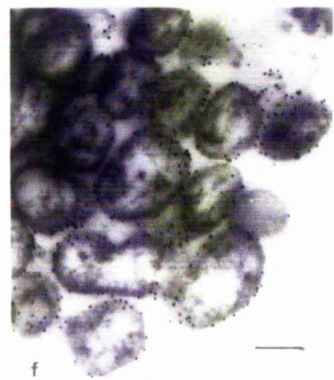
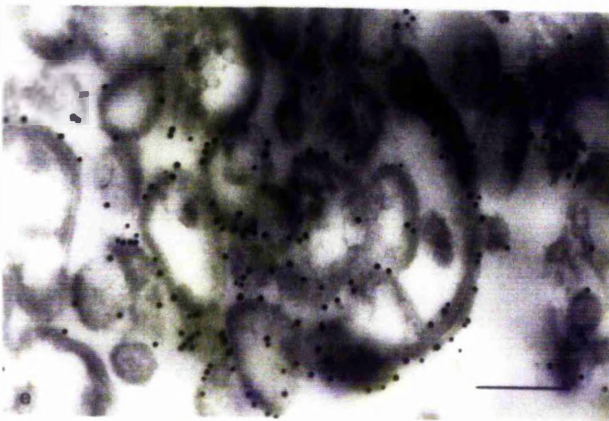
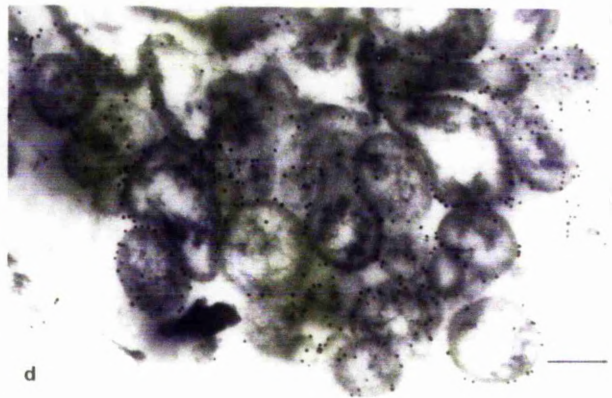
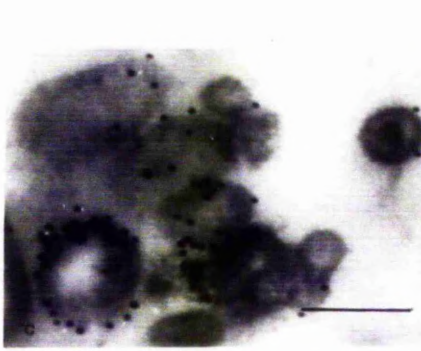
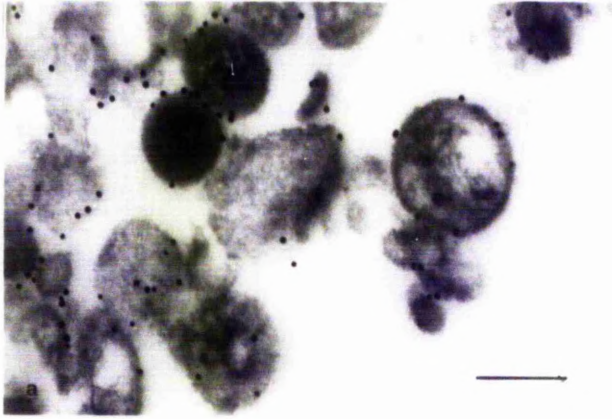


Fig 5.6: Electron micrographs of *U. urealyticum*, serotype 8, stained with an anti-96 kDa Mab UU8/29 and Protein A gold prior to fixation with 0.5 % (v/v) gluteraldehyde in PBS. Bar represents 0.2 μ m.

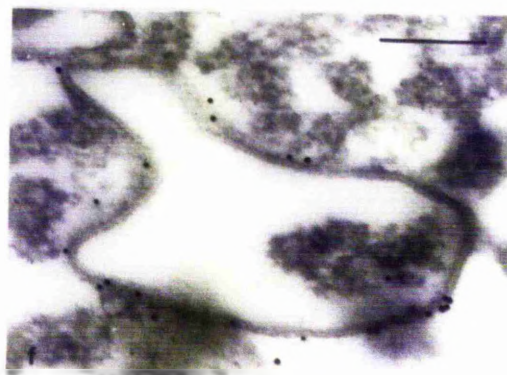
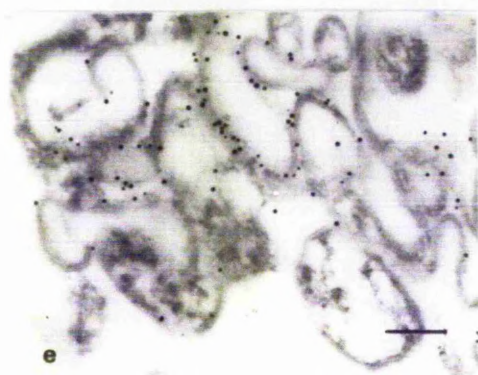
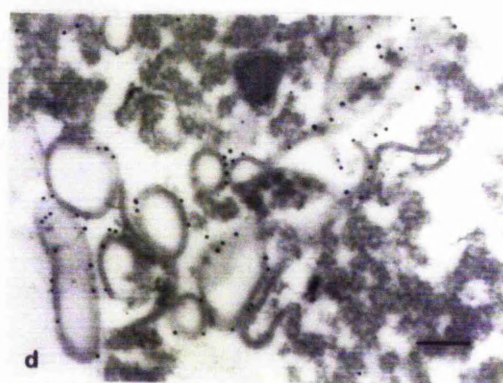
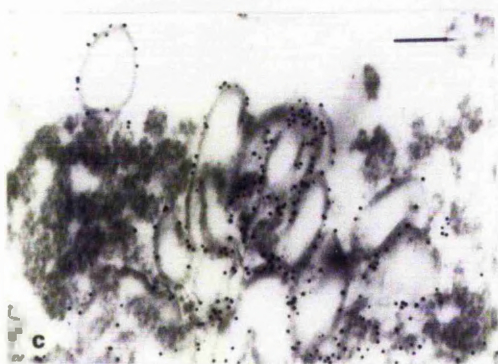
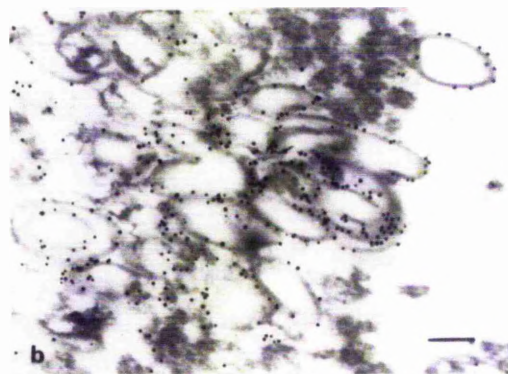
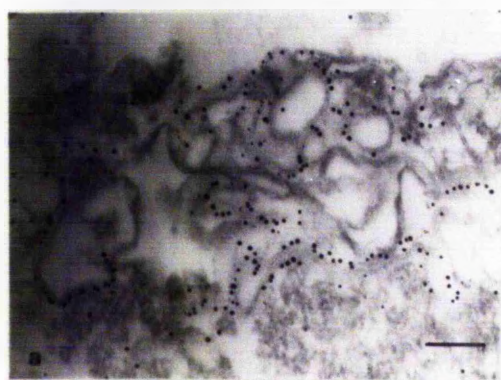
(a) and (e) X 82,000; (b) and (f) X 38,000; (c) X 100,000; (d) X 50,000.



denaturation. The difficulties in purifying this antigen may imply that these polypeptides are integral to the membrane. Thus, cells were gently permeabilised with PBS-N as described (section 2.23) and then labelled as for the 96 kDa antigen, i.e. prior to fixation. The resultant electron micrographs can be seen in Fig 5.7 (a-f). The majority of the cells appeared empty, suggesting that the membrane was permeabilised. However, the majority of membrane 'shells' were very efficiently labelled. Occasionally, whole cells were observed but these were not labelled. The membranes were mostly labelled on what appeared to be the external surface, although labelling did occur on the inner membrane to some extent. This may suggest that the antigenic determinant is surface-expressed but it is not available to the Mab prior to denaturation, sonication or permeabilisation, perhaps due to the close association of other membrane proteins.

Fig 5.7: Electron micrographs of *U. urealyticum*, serotype 8, permeabilised with PBS-N, stained with an anti-16/17 kDa Mab UU8/39 and Protein A gold, prior to fixation with 0.5 % (v/v) gluteraldehyde. Bar represents 0.2 μ m.

(a) and (e) X 49,000; (b) X 35,000; (c) X 42,000; (d) X 41,000; (f) x 82,000.



CHAPTER 6
A COMPARISON OF HUMAN AND NON-HUMAN UREAPLASMAS.

Ureaplasmas have been isolated from many non-human species, with those from cattle (*U. diversum*) receiving the most attention. Those studied here were isolated from chimpanzees (5 strains), cattle (strain T44 used in all tests, 2 other strains used in selected tests only) and from dog, cat and marmoset (1 strain each) (section 2.1). Since all five chimpanzee strains behaved similarly, only one representative strain is shown in the following figures. This was also the case for the three *U. diversum* strains (where all used), and again only one example is shown in each figure.

6.1. THE POLYPEPTIDE PATTERN AS SHOWN BY SDS-PAGE.

The non-human ureaplasmas and *U. urealyticum*, serotypes 1 and 8 were grown and harvested as described in section 2.2. The resultant pellets were sonicated in buffer A (described in section 2.15) and centrifuged at 300 g for 3 minutes to remove the majority of the medium contamination. The resultant electrophoretogram is shown in Fig 6.1 and shows the similarity between both the non-human and human strains with respect to their polypeptide maps. However, the bovine, canine and feline strains were distinct from the human, chimpanzee and marmoset isolates with major differences in region A. It was noteworthy that the canine isolate (lane g) also appears to possess a polypeptide of apparent 96 kDa molecular mass similar to that seen in *U. urealyticum*, serotype 8 (lane c). The canine isolate also appears to have two major antigens of apparent 21 kDa and 18 kDa molecular mass which are not apparent in either the human or other non-human isolates. The feline and canine isolates both appear to have two common polypeptides of molecular masses 40 kDa and 37 kDa.

6.2. THE 96 kDa ANTIGEN.

As discussed in chapter 3, the use of the anti-96 kDa Mabs had shown that so far as *U. urealyticum* was concerned, this 96 kDa antigen is serotype 8-specific. In order to determine whether this antigen was expressed in any of the non-human ureaplasma isolates, these were subjected to SDS-PAGE and immunoblotting where

Fig 6.1: Electrophoretogram of a 15 % (w/v) SDS-polyacrylamide gel of non-human and human isolates stained with Coomassie blue stain (~100 µg protein per track).

Lane A - Molecular mass standards.

Lane B - *U. diversum*.

Lane C - *U. urealyticum*, serotype 8 (representative of serocluster A).

Lane D - *U. urealyticum*, serotype 1 (representative of serocluster B).

Lanes E-H - Ureaplasmas isolated from a:

Lane E - chimpanzee.

Lane F - marmoset.

Lane G - dog.

Lane H - cat.

a b c d e f g h i

$10^3 \times \text{Mol mass (kDa)}$

200.0

97.4

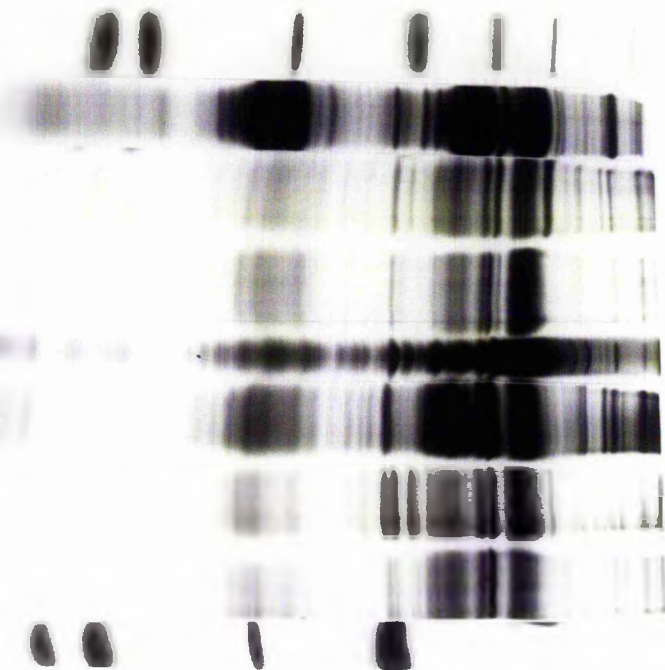
66.0

43.0

25.7

18.4

14.3



the blots were probed with anti-96 kDa Mabs which recognised each of the four known epitopes. One such autoradiogram is seen in Fig 6.2 which shows that the 96 kDa antigen was not detected in any of the non-human ureaplasmas, giving further evidence that the 96 kDa antigen is *U. urealyticum*, serotype 8-specific.

6.3. THE 16 kDa AND 17 kDa ANTIGENS.

The anti-16/17 kDa Mabs and their cross-reactions with the fourteen serotypes of *U. urealyticum* have been documented in chapter 3.

The non-human ureaplasmas and *U. urealyticum*, serotypes 8 and 1 (representative of the two seroclusters) were subjected to SDS-PAGE and immunoblotting with the anti-16/17 kDa Mab (UU8/39). This Mab reacted in an interesting manner, as shown in Fig 6.3. It should be remembered that both the 16/17 kDa polypeptides are recognised in the large serocluster of the human ureaplasmas (lane A) and that the 17 kDa polypeptide only, is recognised in the small serocluster (lane B). As for the human ureaplasmas from the large serocluster, both the 16 kDa and the 17 kDa polypeptides were recognised in the five chimpanzee isolates (lane C). Two polypeptides were also recognised in the ureaplasma isolated from the canine (lane D), but these had modified molecular masses of 16.5 and 17.5 kDa. In the marmoset species (lane E) the 17 kDa polypeptide only was recognised, whereas in the bovine (lane F) and feline species (lane G) of ureaplasmas studied, the 16 kDa polypeptide only was recognised. Thus it would appear that the 16 kDa and 17 kDa polypeptides, either together, singly, or in a slightly modified state, are conserved across all the ureaplasma species studied here and this may suggest an important role for these polypeptides. The metabolic inhibition studies however, suggest that these polypeptides are not important in the metabolism of this organism (chapter 3). Thus, they may perform a structural role in the membrane. However, due to the apparent inaccessability of these antigens to the Mabs (chapter 3) the results from the metabolic inhibition studies may be misleading.

Fig 6.2: Immunoblot of five non-human ureaplasmas and *U. urealyticum*, serotype 8, probed with an anti-96 kDa Mab (UU8/29) after electrophoretic separation on a 15 % (w/v) SDS-polyacrylamide gel (~ 100 µg protein per track).

Lane A - *U. urealyticum*, serotype 8.

Lane B - *U. diversum*.

Lanes C-F - Ureaplasmas isolated from a:-

Lane C - Chimpanzee.

Lane D - Marmoset.

Lane E - Dog.

Lane F - Cat.

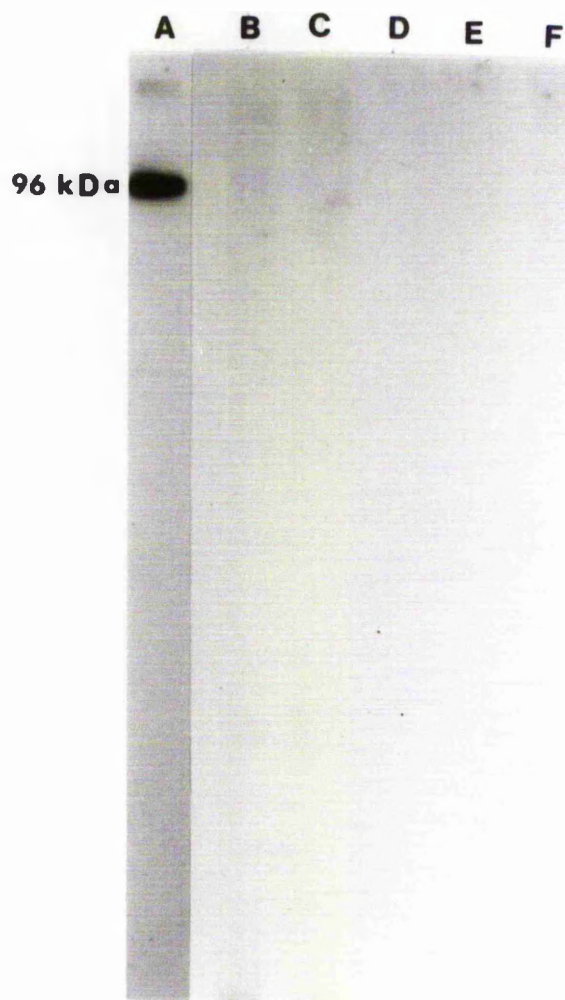


Fig 6.3: Immunoblot of

Lane A - *U. urealyticum*, serotype 8.

Lane B - *U. urealyticum*, serotype 1.

Lanes C-G - Ureaplasmas isolated from a:-

Lane C - Chimpanzee.

Lane D - Dog.

Lane E - Marmoset.

Lane F - Cow, i.e. *U. diversum*.

Lane G - Cat.

All tracks were probed with an anti-16/17 kDa Mab (UU8/39) and ^{125}I -labelled protein A after electrophoretic separation on a 15 % (w/v) SDS-polyacrylamide gel (~ 100 μg protein per track).

A B C D E F G



It was noteworthy that the results from the chimpanzee isolates and the marmoset isolates were similar to the large serocluster and the small serocluster of *U. urealyticum* respectively.

Whether these polypeptides are two separate polypeptides with a common epitope or are related by modification is unknown.

6.4. THE UREASE ENZYME.

Like the human ureaplasmas, the non-human ureaplasmas have a requirement for urea and possess a urease enzyme; this enzyme has been discussed in detail in chapter 4. The urease enzymes of the non-human strains and their cross-reaction with the anti-urease Mabs, raised against serotype 8, were studied.

6.4.1. POLYACRYLAMIDE GEL ELECTROPHORESIS.

U. urealyticum, serotypes 1, 5, 9 and 13, being representatives of the human ureaplasmas, and the non-human strains were subjected to non-denaturing PAGE (section 2.6.2) (Fig 6.4 (a)) and to denaturing SDS-PAGE (section 2.6.1), (Fig 6.4 (b)), prior to staining with lead acetate (section 2.17 (2)) (as discussed previously, section 4.5). Again whereas both gels show a similar pattern, the ureases appear as two active bands on the SDS-denaturing gel compared to only one active band on the 'native' gel system. Similarly, it is possible that the lower molecular weight band showing the least activity represents a slightly denatured form of the urease

As described for the ureases of the human serotypes (section 4.5) the molecular masses of the ureases from the non-human strains also display molecular mass variation.

The non-human ureaplasmas were also probed with the anti-urease Mab (UU8/17) after SDS-PAGE and semi-dry blotting to nitrocellulose. The resultant autoradiogram

Fig 6.4: Non-human and human ureaplasmas subjected to:-

(a) Non-denaturing (7.5 % w/v) polyacrylamide gel electrophoresis.

(b) Denaturing (7.5 % w/v) polyacrylamide gel electrophoresis.

Stained with lead acetate (~100 ug protein per track).

A: Lane a - Ureaplasma isolated from a cat.

Lane b - *U. diversum*

Lane c - Ureaplasma isolated from a dog.

Lane d - Ureaplasma isolated from a marmoset.

Lane e - Ureaplasma isolated from a chimpanzee.

Lanes f-i - *U. urealyticum*, serotypes 9,13,1 & 5 respectively.

B: Lane a - *U. urealyticum*, serotype 5.

Lane b - Ureaplasma isolated from a chimpanzee.

Lane c - Ureaplasma isolated from a marmoset.

Lane d - Ureaplasma isolated from a dog.

Lane e - *U. diversum*

Lane f - Ureaplasma isolated from a cat.

Lanes g-i - *U. urealyticum*, serotype 1,13 & 9 respectively.



Fig 6.5: Electrophoretic and immunoblot analysis on a 15 % (w/v) SDS-polyacrylamide gel of denatured urease, probed with anti-urease Mab UU8/17.

Lane a - *U. urealyticum*, serotype 8.

Lane b - *U. diversum*.

Lanes c-f - Ureaplasmas isolated from a:-

Lane c - cat.

Lane d - dog.

Lane e - marmoset.

Lane f - chimpanzee.

a b c d e f

72 kDa

(Fig 6.5) shows that the inactive 72 kDa subunit of the urease was recognised in the feline, marmoset and chimpanzee isolates but not in those from the bovine or canine isolates. It should be remembered that this subunit is also not detected on immunoblots of *U. urealyticum*, serotypes 3, 6 and 14 with this Mab (see chapter 4).

As discussed previously, Mabs were raised which recognised at least four, perhaps five, distinct epitopes on the urease enzyme of serotype 8 (chapter 4). However, using the urease 'catch test' (section 2.21) and *U. diversum* strains, the results suggested that the anti-urease Mab (UU8/1), which may have belonged to epitope group A (chapter 4), behaved differently, in that the Mab did not recognise the urease from *U. diversum*, unlike the other anti-urease Mabs in this group. This therefore suggests the presence of at least five epitope groups on the enzyme, and agrees with the results obtained using the second competition assay method. Presumably all these epitopes are away from the active site since binding of any of these Mabs does not inhibit enzymic activity.

The wells of a 96-well plate were coated with an anti-urease Mab from each of the five epitope groups and the catch assay was carried out with each of the non-human strains and with *U. urealyticum*, serotypes 1-14. The results of this assay are shown in Fig 6.6 and are tabulated below in Table 6.1.

Fig 6.6: Urease catch assay using logarithmic cultures ($\sim 10^7$ c.c.u. ml^{-1}) of:-

Row A: *U. urealyticum*, serotype 8.

Rows B-E: Ureaplasmas isolated from a:-

Lane B - marmoset.

Lane C - chimpanzee.

Lane D - dog.

Lane E - cat.

Lane F - *U. diversum*.

The plate was precoated with a 1/200 dilution of GAM in PBS.

Lane 1 - Blank (no antigen added).

Lanes 3-12 - coated with anti-urease Mabs from the five different epitope groups.

Lanes 3 + 4 - UU8/17.

Lanes 5 + 6 - UU8/16.

Lanes 7 + 8 - UU8/12.

Lanes 9 + 10 - UU8/25.

Lanes 11 + 12 - UU8/1.

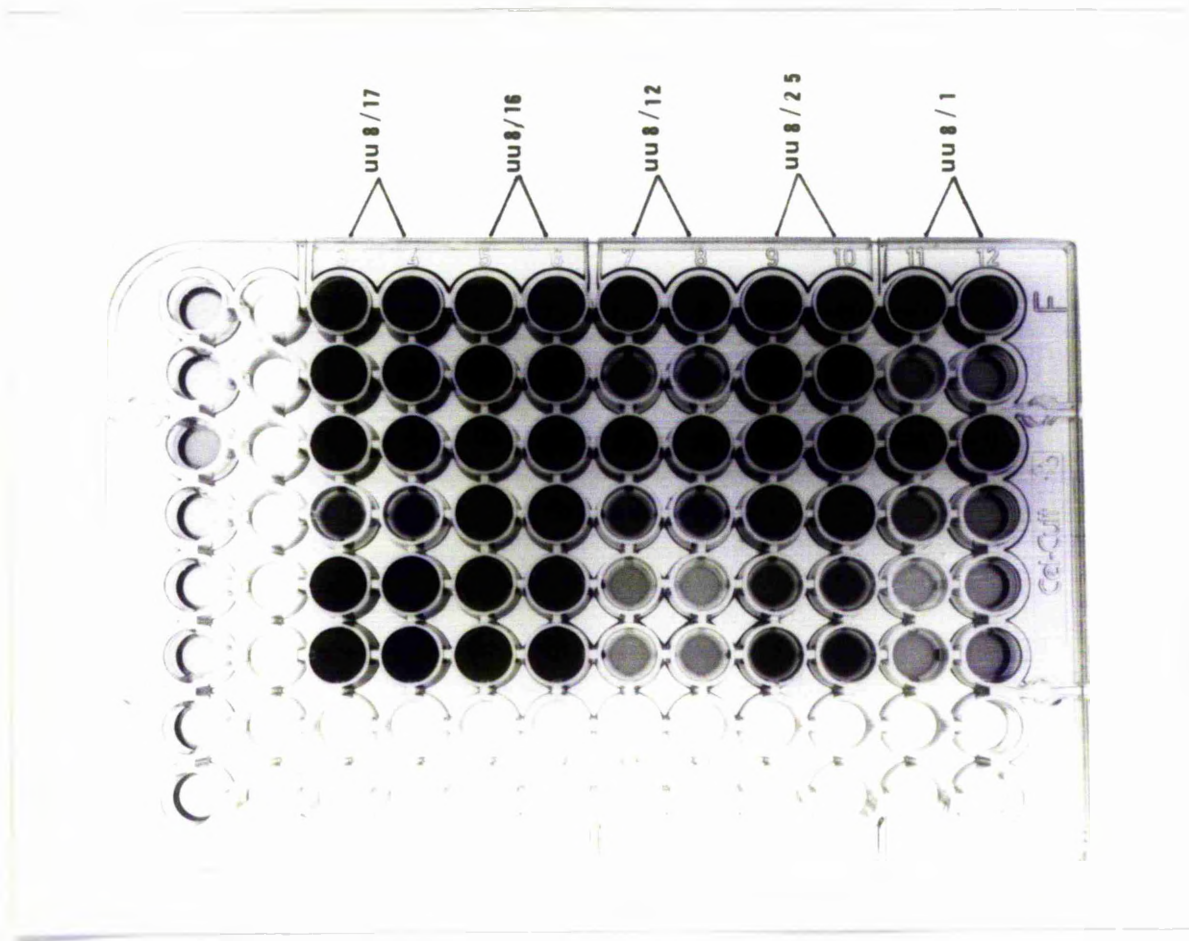


Table 6.1: Summary of urease catch test results.

Source of urease		Urease epitope groups 1-5 represented by:-				
		UU8/17	UU8/16	UU8/12	UU8/25	UU8/1
A	<i>U. urealyticum</i> (serotypes 1-14)	+++	+++	+++	+++	+++
B	Marmoset	+++	+++	+	+++	-
C	Chimpanzee (strains 1-5)	+++	+++	+++	+++	+++
D	Canine	+	+++	+	++	-
E	Feline	+++	+++	-	+	-
F	<i>U. diversum</i> (strains 1-3)	+++	+++	+	++	-

+++ - represents strong colour reaction (Absorbance between 1.2-1.99 at 600nm)

++ - represents medium colour reaction (Absorbance between 0.6-1.2 at 600nm)

+ - represents weak colour reaction (Absorbance between 0.1-0.6 at 600nm)

- - represents no colour reaction (Absorbance less than 0.1 at 600nm)

It can be seen that only the chimpanzee isolates behave similarly to the human serotypes, suggesting that the chimpanzee urease possesses and has available, the five epitopes of the *U. urealyticum* urease. Whereas the ureases of the ureaplasmas isolated from cattle and marmoset reacted similarly in this test this is not true for any of the other studies with the urease enzyme.

The different reactions of the non-human ureaplasmas with the anti-urease Mabs confirm that there are differences in the structure and/or organisation of the urease from different strains such that epitopes are altered or are unavailable for binding and this confirms the results from the lead acetate-stained gels.

Many workers feel that the chimpanzee may be the only natural animal model for the study of *U. urealyticum* pathogenicity and it is interesting to note that with the exception of the 96 kDa antigen, the five chimpanzee strains alone gave identical results to those serotypes of *U. urealyticum* belonging to the larger serocluster. This

confirms the view of Mouches *et al.* (1981), who suggest that ureaplasmas of the chimpanzee are more closely related to *U. urealyticum* serocluster A than any other animal ureaplasmas.

With the exception of the 'urease catch test' results, the marmoset strains gave similar results to those *U. urealyticum* serotypes belonging to the small serocluster. Mouches *et al.* (1981) also suggested that marmoset ureaplasmas were distinct from those of other animal species.

CHAPTER 7
DIAGNOSTIC TESTS.

As discussed in chapter 1, ureaplasmas are not screened for routinely in most laboratories because the majority of tests require the growth of the organism either on solid or in liquid media, which is time consuming and costly. Growth in liquid media is quicker and is usually more sensitive (Taylor-Robinson *et al.*, 1969), but growth on solid media is often required to confirm the results as many of the tests employed (section 1.1) (e.g. immunofluorescence) require the growth of the organism on solid media.

Diagnostic tests which are sensitive, easy to perform and which do not require the growth of these fastidious organisms would greatly improve the situation in terms of rapid diagnosis, of defining the role of *U. urealyticum* in the pathogenicity of man and also in terms of patient treatment. This chapter discusses the development of four such tests.

7.1. FLUORESCENCE TECHNIQUES.

7.1.1. INDIRECT METHOD.

The main advantage of this method is that only one fluorescent probe which recognises the primary antibody is required.

U. urealyticum, serotype 8, was probed with an anti-96 kDa Mab (UU8/29) and a rhodamine-linked goat anti-mouse immunoglobulin, as described in section 2.24.1. *U. urealyticum*, serotype 7, *M. hominis* and *M. ovipneumoniae* probed as above and *U. urealyticum*, serotype 8, probed with an anti-viral Mab were used as negative controls. Using this technique, bright red fluorescence was seen with serotype 8 and Mab UU8/29, but background fluorescence was observed with the negative controls, even after thorough washing and pre-adsorption of the rhodamine conjugate with sonicated ureaplasma cell extract.

7.1.2. DIRECT METHOD.

This method of staining (section 2.24.2) was elected for further study because of the problems of background fluorescence discussed above.

Mabs which recognise the urease, the 96 kDa and the 16/17 kDa antigens were purified (section 2.10) and labelled with fluorescein isothiocyanate (FITC) as described (section 2.24.2).

7.1.3. ANTI-96 kDa AND ANTI-16/17 kDa MABS.

The anti-96 kDa and anti-16/17 kDa Mabs have been described in detail in chapters 3 and 5. Results suggest that these Mabs recognise surface expressed membrane antigens, but that the 16/17 kDa antigens were more readily available to the Mab after denaturation, sonication or permeabilisation; thus, cell extracts were permeabilised with PBS-N prior to staining (section 2.24)).

For comparison of immunofluorescence staining, black and white photographs are shown, as the tone in the colour photographs tended to vary due to problems with exposure. Negative controls were as described above.

Fig 7.1 (A,C) shows *U. urealyticum*, serotype 8 (A - 0.5 mg protein ml⁻¹; C - 0.1 mg protein ml⁻¹), probed with the FITC-linked anti-96 kDa Mab (UU8/29). Fig 7.1 (B,D) shows the equivalent dilutions with *M. hominis*, probed as above, and although material could be seen on the slide, this did not fluoresce. Cell extracts of *M. ovipneumoniae* and *U. urealyticum*, serotype 7 gave similar results to those seen with *M. hominis* (results not shown), again confirming the serotype 8-specificity of the anti-96 kDa Mabs.

Fig 7.2 (A), shows *U. urealyticum*, serotype 8 (0.5 mg protein ml⁻¹), probed with the FITC linked anti-16/17 kDa Mab (UU8/39) and a similar result was seen with serotype

Fig 7.1: A/C – *U. urealyticum*, serotype 8 probed with an FITC-anti-96 kDa Mab UU8/29.

B/D – *M. hominis* probed as for A/C.

A,B – 0.5 mg protein ml⁻¹.

C,D – 0.1 mg protein ml⁻¹.

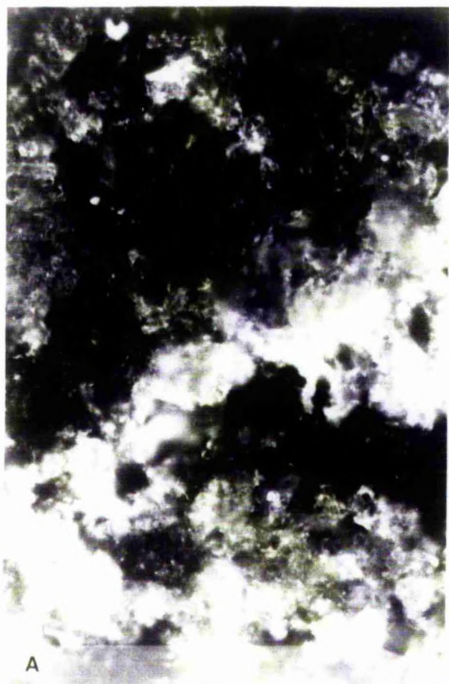
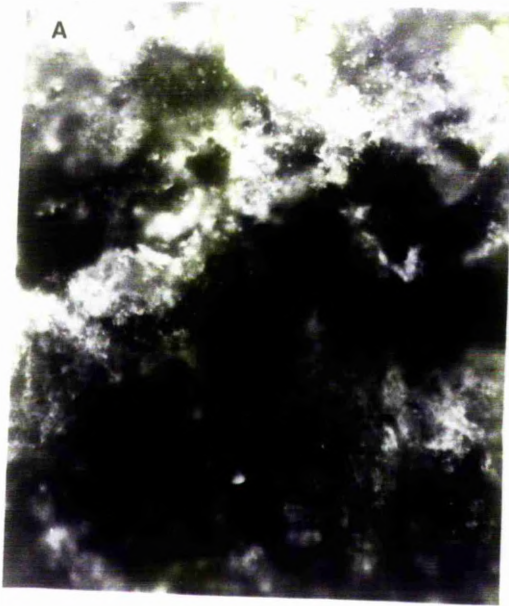


Fig 7.2 (A): *U. urealyticum*, serotype 8 (0.5 mg protein ml⁻¹) probed with FITC-anti-16/17 kDa Mab UU8/39.

(B): *M. hominis* (0.5 mg protein ml⁻¹) probed as in (A).



7. *M. hominis* (0.5 mg protein ml⁻¹) probed as described above is shown in Fig 7.2 (B) and Fig 7.3 (A,B) shows the colour equivalent of Fig 7.2 (A,B).

Bright green fluorescence (Fig 7.3 (A)) was seen with both FITC-linked Mabs and the appropriate antigen. However, the staining observed with the anti-96 kDa Mab was more intense than that seen with the anti-16/17 kDa Mab, which may reflect the number of molecules of the 96 kDa antigen available for binding of the Mab, again suggesting this antigen is a major antigen of serotype 8. The 16/17 kDa antigen may be present in the same molar ratio but may be unavailable to the Mab as discussed previously, even though permeabilisation was carried out to try and overcome this problem.

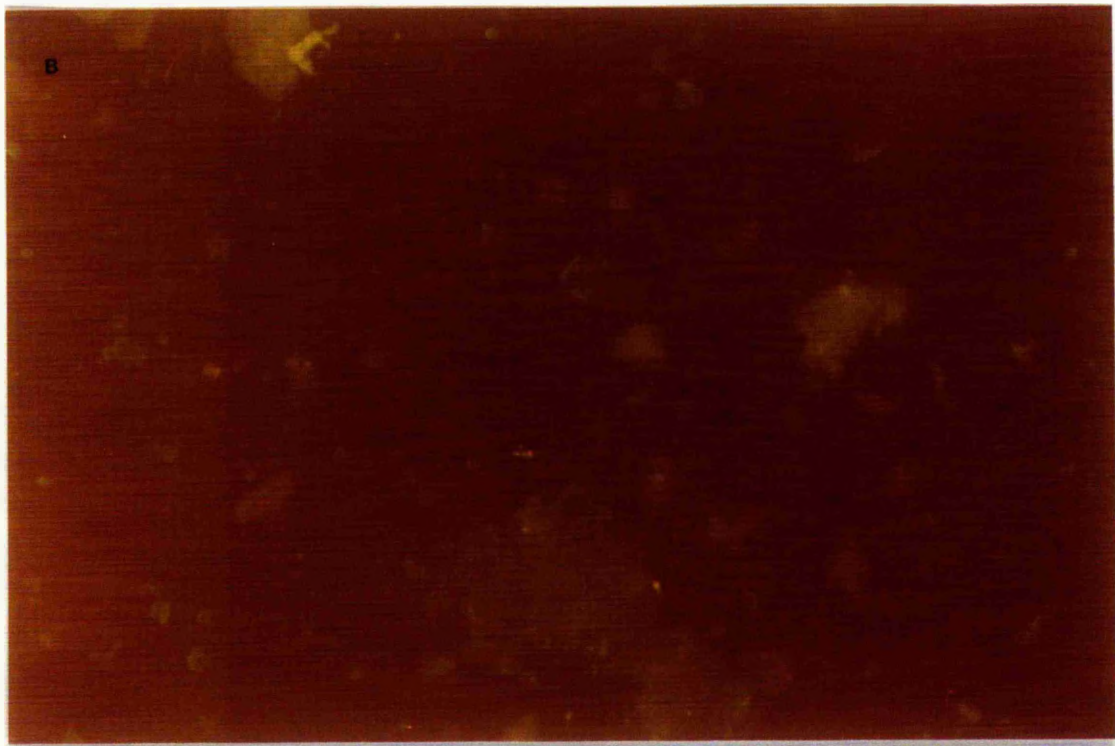
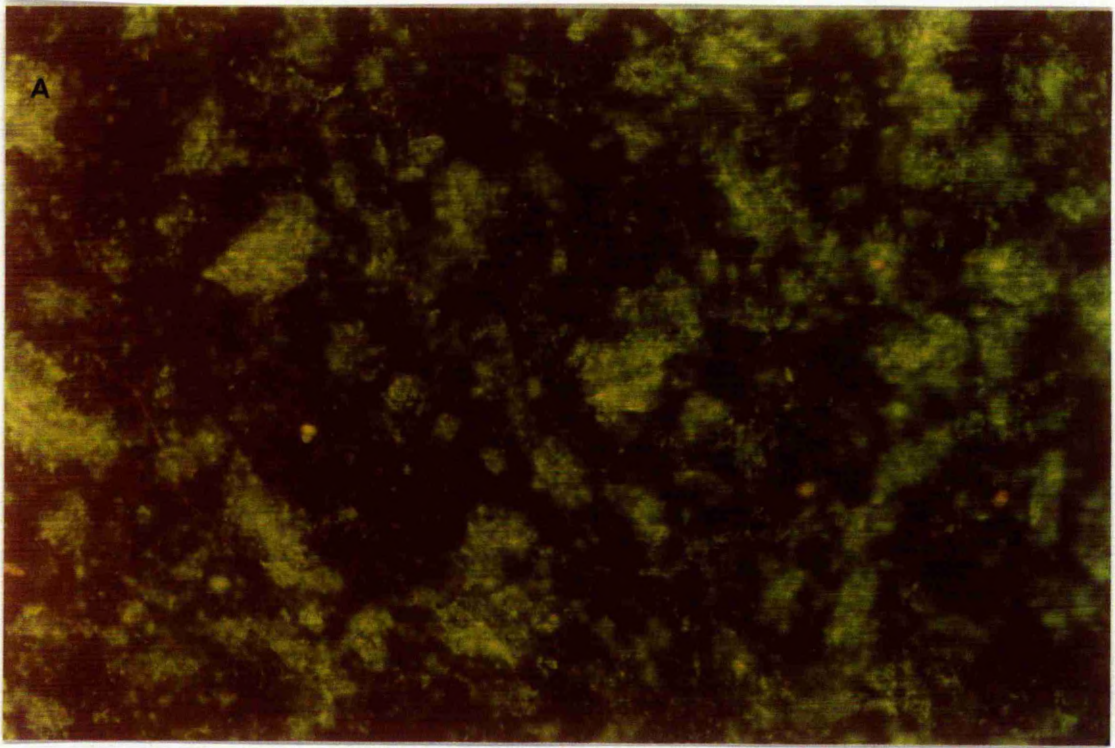
7.1.4. SENSITIVITY OF THE FITC-CONJUGATED MABS.

The sensitivity of these probes was tested in two ways as described in section 2.24.3.

(1) Results from a 10-fold dilution series from a log culture of *U. urealyticum*, serotype 8 suggested that each Mab could detect down to 10³ c.c.u. ml⁻¹ i.e. 10¹ organisms. However, it was difficult to differentiate this fluorescence from small 'dots' of background fluorescence, thus a second method was studied to confirm these results.

(2) Both baby hamster kidney (BHK) and HeLa cells were incubated with *U. urealyticum*, serotype 8 as described (section 2.24.3). No fluorescence was seen associated with the BHK cells, suggesting that ureaplasmas cannot adsorb to these cells. Fluorescence was however, seen associated with the HeLa cells and this technique also suggested that these probes could detect organisms from a culture containing 10³ c.c.u. ml⁻¹, i.e. 50 organisms (as 50 µl samples were used instead of the normal 10 µl samples).

Fig 7.3 (A + B): As described for Fig 7.2.



7.1.5. ANTI-UREASE MABS.

The urease enzyme and the anti-urease Mabs have been described in detail in chapter 4 and it should be remembered that this enzyme is localised in the cytoplasm of the organism.

U. urealyticum, serotypes 8 and 7 (0.5 mg protein ml⁻¹) were probed with FITC-linked anti-urease Mabs UU8/17 and UU8/1 as described in section 2.24.2 and one such result is shown in Fig 7.4 (A). *M. hominis*, (0.5 mg protein ml⁻¹), probed as above is shown in Fig 7.4 (B), as a negative control, where no fluorescence was observed and a similar result was seen with *M. ovipneumoniae*

The fluorescence observed with both anti-urease Mabs was not as bright or as intense as that seen with the anti-96 kDa or anti-16/17 kDa Mabs and this may be because the urease is cytosolic and may not have been readily available to the Mab even after permeabilisation. Also, as the urease is soluble, it is possible that the majority of the enzyme was removed during washing procedures.

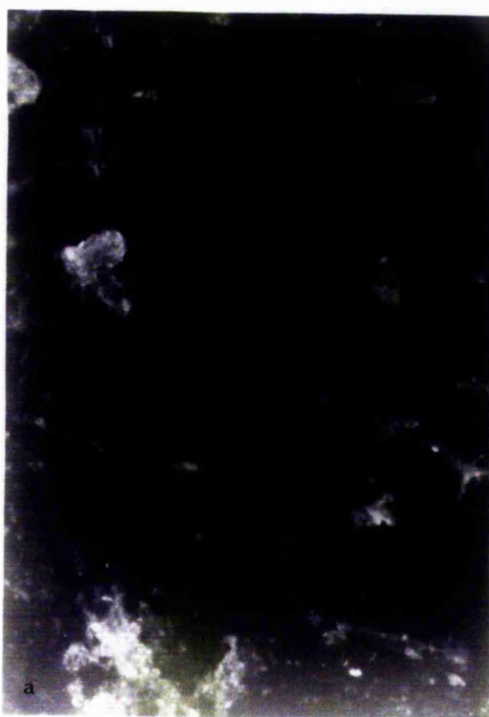
7.2. THE UREASE CATCH ASSAY.

This assay has been discussed in chapter 4 and section 2.21 and several assays are shown in chapter 4. In the laboratory, using log cultures of *U. urealyticum*, this test was shown to have a sensitivity down to 10³ c.c.u. ml⁻¹ (section 2.21), i.e. detection of 10² organisms

Urethral clinical samples, in duplicate, were made available from Ninewells Hospital in the form of calcium alginate swabs (section 2.25). One of each pair of these swabs was placed in Stuart's transport medium and processed as described in section 2.22 by the Ninewells staff and the following results were obtained:

Fig 7.4 (A): *U. urealyticum*, serotype 8 probed with FITC-anti-urease Mab UU8/17 (0.5 mg protein ml⁻¹).

(B): *M. hominis* (0.5 mg protein ml⁻¹) probed as in (A).



Total number of samples examined	91
Total number of ureaplasma isolations	8
Dual isolation of ureaplasma/ <i>M. hominis</i>	1

Thus in total, ureaplasmas were only isolated in 9.9 % of cases which was much lower than expected as the majority of these patients were male and aged under 50 where the expected isolation rate was at least 21 % (chapter 1). This low isolation rate could be due to several reasons:-

(1) It has been suggested that calcium alginate swabs may contain inhibitors for ureaplasmas and they should be agitated in the transport medium and immediately removed (Taylor-Robinson, 1983b); this was not done.

(2) Stuart's transport medium is not the ideal medium for transportation of ureaplasmas and more organisms may have survived had growth medium minus urea, been used.

(3) Samples should be processed as soon as possible and within 24 hours during which time they should be stored at 4°C. In many cases this did not occur as samples taken on a Friday were often left until Monday before being cultured. In three cases, samples were stored at 37°C for several hours before being processed.

(4) The solid medium used (section 2.25) contained thalious acetate to which ureaplasmas are sensitive (Lee *et al*, 1972) and this may have at least partially inhibited the growth of these organisms.

Duplicate swabs were frozen in PBS at -70°C and processed as described (section 2.25), but all were negative when assayed using the catch test. The calcium alginate swabs which were used should have dissolved which would have increased the

chances of detecting this organism. However, the swabs only partially dissolved to form a thick jelly-like substance which was very difficult to handle and any ureaplasmas caught in the matrix of the swab would not have been sampled.

Unfortunately, no quantitative data was available for these samples and low numbers of organisms may also have contributed to the negative results. As this test does not require viable organisms, the presence of any inhibitors to the organism but not to the urease in the swab should not have affected the results. This was studied when dilutions of ureaplasma seed cultures prepared in the laboratory in the presence of alginate swabs gave comparable positive results to samples prepared in the absence of swabs.

Frozen urine samples (section 2.25 (b)) were processed as described and negative results were again obtained with the catch assay. Attempts were also made to culture these samples in liquid growth medium but these were also negative.

Quantitative data for these 48 samples were obtained from Dr. D. Taylor-Robinson (CRC, Harrow) and these data are shown below:-

Total number of negative urine samples	31
Number of urine samples initially shown to contain:-	
10^1 <i>U. urealyticum</i> c.c.u. ml ⁻¹	7
10^2 <i>U. urealyticum</i> c.c.u. ml ⁻¹	6
10^3 <i>U. urealyticum</i> c.c.u. ml ⁻¹	3
10^4 <i>U. urealyticum</i> c.c.u. ml ⁻¹	1
Total urine samples positive for <i>U. urealyticum</i>	17 (35 %)

The isolation rate of 35 % was much higher than expected (see previously) but this may have been due to the fact that these samples were cultured immediately they were available.

Tarr *et al.* (1976) have suggested that ureaplasmas are isolated less frequently from urine samples than from urethral swabs, but this was not shown here. However, direct comparison cannot be made since the urethral and urine samples were cultured in/on different media. As discussed above, liquid medium is thought to be more sensitive and, the higher isolation rate seen with the urine samples may be due to the fact that they were cultured in such a liquid medium.

Since the catch assay has a sensitivity of 10^3 c.c.u. ml⁻¹, i.e. 10^2 organisms one would have expected that positive results would have been seen with at least 4 of the 48 urine samples. However, freezing the organisms in urine was found to reduce the urease activity at least 10 fold and in addition, freezing the organisms in urine was shown to reduce the viability of the organisms 100 fold and these factors would explain the negative results on culture.

7.3. DIP-STICK TEST.

This test (described in section 2.26) is very similar to the urease catch assay in that it also "catches" the urease enzyme of *U. urealyticum* and after metabolism of the urea to ammonia, this is detected by the Berthelot reaction.

However, using 100 μ l samples of diluted seed culture, the test was shown to be 10-fold less sensitive i.e. the lowest seed dilution from which a positive result was obtained was one containing 10^4 c.c.u. ml⁻¹ or 10^3 organisms. Nevertheless, whereas the urease catch test assay can use only 100 μ l of test sample, the dip-stick can be placed in any volume of sample and if urease is present, it will bind to the Mab and be detected as described above. However, contrary to expectation, when 10^3 organisms were diluted in 20 mls of PBS-N, no positive result was obtained. On dilution to this much larger volume, the minimum detection limit with the dip-stick was found to be 10^4 organisms. This may be a reflection of the ability of the small surface area of the coated nitrocellulose relative to the total volume to bind all the

urease within a reasonable time period.

The urine samples described in section 7.2 were also tested using the dip-stick, but unfortunately these results were again negative. This may have been due to the decrease in urease activity caused by freezing in urine, as observed in the control investigations.

7.4. SLIDE AGGLUTINATION.

Slide agglutination tests make use of a solid matrix to which a specific anti-serum is adsorbed. When this Ab-matrix is mixed with a suspension of homologous antigen, a lattice is formed which causes agglutination to occur which can be observed visually without the use of a microscope. This reaction usually occurs within minutes and thus this test is very simple and easy to carry out.

The most common solid matrix used is *Staphylococcus aureus* (Cowan strain), which is formaldehyde and heat-treated to inactivate the enzymes which cause autolysis (Kronvall, 1973). The cell wall of *Staphylococcus aureus* contains Protein A which has a high affinity for the Fc portion of immunoglobulin G (Forsgren and Sjoquist, 1966) and thus the Fab structure of the antibody is orientated outwards and available for binding of the antigen. This test is used routinely, for example, using anti-serum raised against *Streptococcus pneumoniae* to successfully type pneumococci isolates (Kronvall, 1973; Christiansen et al., 1973).

Mabs, both in a purified and non-purified state and at various dilutions, which recognise the urease, the 96kDa and the 16/17 kDa antigens (described in chapters 3 and 4) were successfully adsorbed to a suspension of *Staphylococcus aureus* as described in section 2.27 as shown by SDS-PAGE and coomassie blue staining which revealed the presence of both light and heavy immunoglobulin chains (data not shown).

The suspension of *Staphylococcus aureus*, without the presence of antibody was mixed as follows:-

(1) on its own, (2) with PBS, (3) with growth medium and (4) with a cellular extract of *U. urealyticum*, serotype 8 (1 mg protein ml⁻¹) and the results are shown in Fig 7.5 A (a). Whilst the suspension of *Staphylococcus aureus* did not co-agglutinate on its own or in the presence of PBS (1&2), in the presence of growth medium or ureaplasma cell extract (3&4) some signs of agglutination were apparent.

The results using the *Staphylococcus aureus*-anti-urease Mab (UU8/17) and the *Staphylococcus aureus*-anti-16/17 kDa Mab (UU8/39) complexes alone and with the above three treatments are shown in Fig 7.5 A (b and c respectively). Again, no agglutination was seen with the conjugates on their own or with PBS, but agglutination was seen with growth medium and with ureaplasma cell extract. Similar results were seen when using *Staphylococcus aureus*- anti-96 kDa Mab complexes.

Due to the agglutination observed with the growth medium and *Staphylococcus aureus* complexes, a different solid matrix, latex beads, was studied.

7.5. LATEX BEADS.

After ensuring no self-agglutination occurred with these beads (results not shown), the Mabs, in a purified state, and at various dilutions were adsorbed to the particles as described in section 2.27 and that this had been achieved successfully was checked using SDS-PAGE and coomassie blue staining. Each Mab-latex reagent was treated as for the *Staphylococcus aureus* complexes and the results are shown in Fig 7.5 B (a - d). No agglutination was seen using an anti-16/17 kDa Mab at a dilution of 1/10 (a) or at 1/100 (b), nor was any seen using an anti-96 kDa Mab (UU8/29) undiluted (5mg protein per ml)(c) or using a 1/10 dilution of an anti-urease Mab (UU8/17)(d). The reasons for this are unknown but the SDS-PAGE and coomassie blue staining suggested that only a small amount of antibody adsorbed to these particles which

Fig 7.5 (A): Slide Agglutinations using *Staphylococcus aureus* complexed:-

(a) alone.

(b) with an anti-urease Mab (UU8/17).

(c) with an anti-16/17 Mab (UU8/39).

Treated as follows:-

(1) mixed alone, (2) mixed with PBS, (3) mixed with growth medium and (4) with a cellular extract of *U. urealyticum*, serotype 8 (1 mg protein ml⁻¹).

Fig 7.5 (B): Slide agglutinations using latex beads (0.2 μ m in diameter) complexed:-

(a) with 1/10 dilution in PBS of anti-16/17 kDa Mab UU8/39.

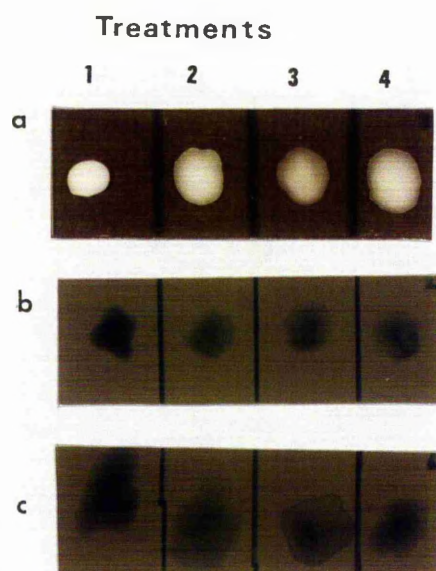
(b) with 1/100 dilution in PBS of anti-16/17 kDa Mab UU8/39.

(c) with undiluted anti-96 kDa Mab UU8/29.

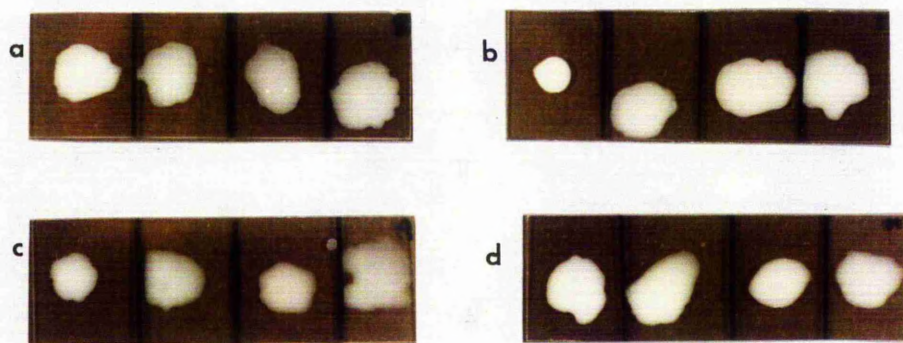
(d) with 1/10 dilution in PBS of an anti-urease Mab UU8/17.

Treated 1-4 as in Fig 7.5 (A).

A



B



could be due to the small size (0.2 μm) of these particles. If only one or two antibodies bound to each particle then the formation of a lattice would be unfavourable. It should also be noted that if, as reported here and elsewhere (chapters 4 and 5) that the urease is cytosolic, then no agglutination would be expected to occur using an anti-urease Mab.

7.6. DYNOSPHERES.

A third type of matrix was used which was a gift from Roy Edwards of Dyno Particles. These particles have a core and shell structure and the shell is composed of an acrylic co-polymer which can be activated for the covalent coupling of proteins such as antibodies (see section 2.27). These particles were supplied in two sizes, 10.4 μm and 3 μm .

Fig 7.6 A (a,b) shows the results obtained with 10.4 μm dynospheres both on their own (a) and coupled to the anti-16/17 kDa Mab (UU8/39) (b), treated 1-4 as above. Unfortunately, due to the large size of these particles they settled out during gentle agitation and were thus unsuitable for such a test procedure.

Fig 7.6 B (a,b) shows results obtained with the 3 μm dynospheres, both on their own (a) and coupled to the anti-16/17 kDa Mab (b). Although these particles did not settle out, neither did they agglutinate. However, when these particles were coupled to an anti-96 kDa Mab (UU8/29) good specific agglutination was observed (Fig 7.7). To ensure this agglutination was specific for *U. urealyticum*, *M. hominis* was also tested as described above and no agglutination was observed as shown in Fig 7.7 (b).

A log culture of *U. urealyticum*, serotype 8 was used to test the sensitivity of this test but even at a titre of 10^8 c.c.u. ml^{-1} no agglutination was seen suggesting that either the test is of low sensitivity or that aggregates of organisms had occurred during pelleting and resuspension of the test culture which enabled visualisation of the lattice.

Fig 7.6 (A): Slide agglutinations using dynospheres (10.4 μm in diameter) complexed:-

(a) alone.

(b) with an anti-16/17 kDa Mab UU8/39.

Treated 1-4 as before.

Fig 7.6 (B): Slide agglutinations using dynospheres (3 μm in diameter) complexed:-

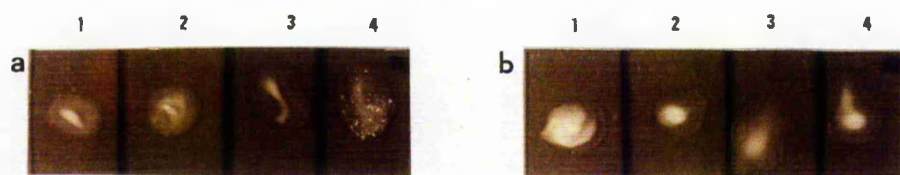
(a) alone.

(b) with an anti-16/17 kDa Mab UU8/39.

Treated 1-4 as before.

A

Treatments



B

Treatments

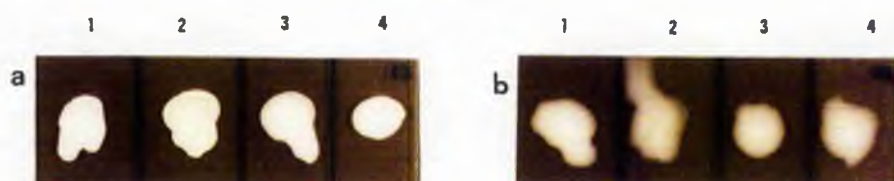
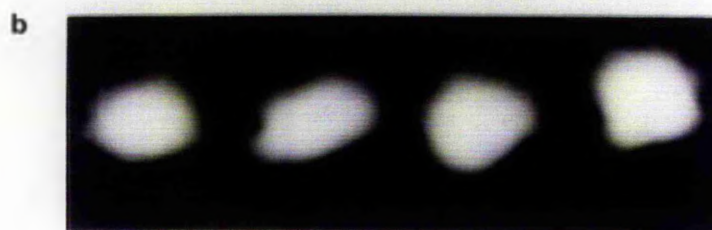


Fig 7.7: Slide agglutinations using dynospheres (3 μm in diameter) complexed with an anti-96 kDa Mab UU8/29.

(a) mixed with 10-fold dilutions of *U. urealyticum*, serotype 8, in PBS at a starting concentration of 10 mg protein ml^{-1} .

(b) mixed with 10-fold dilutions of *M. hominis* in PBS at a starting concentration of 10 mg protein ml^{-1} .



The fact that no agglutination was observed using the anti-16/17 kDa Mab suggests that either these antigens are not accessible to the Mab without prior sonication or permeabilisation or are present at low copy number.

CHAPTER 8
DISCUSSION.

After SDS-PAGE, a minimum of 35–40 polypeptides were clearly visualised in all 14 serotypes of *U. urealyticum* using the sensitive silver stain. In general, the patterns were very similar but some differences were seen both with respect to individual polypeptides which may be serotype-specific, and with respect to the quantitative amounts of individual polypeptides. The problem of protein contamination from the growth medium was reduced by growing the organisms in a medium containing foetal calf serum (Precious *et al.*, 1987). Howard *et al.* (1981) and Mouches *et al.* (1981) circumvented this problem by labelling the organisms with ³⁵S-labelled methionine, but work in this laboratory suggested only poor incorporation was achieved (Precious, personal communication). It is possible that the conditions used by Precious were sub-optimal for incorporation. Again, approximately 40 polypeptides were visualised but it should be noted that some polypeptides may be devoid of this amino acid.

The close similarities shown among the serotypes indicate that they are very closely related and that the original decision, based on genetic homogeneity, to divide these organisms into types rather than species was probably correct.

As noted by Sayed and Kenny (1980), of the 35–40 polypeptides visualised, most strains had at least one unique major polypeptide. Whether these unique polypeptides correlate with the antigenic determinant responsible for serotype differences remains to be elucidated. Using this single dimension electrophoretic technique it was not possible to substantiate the claim of Mouches *et al.* (1981) that from the differences in polypeptide patterns, it is possible to divide the 14 serotypes into the 2 seroclusters. However, whether this can be achieved using 2-dimensional gel electrophoresis, as reported by Swenson *et al.* (1983), was not studied here.

The predominance of Mabs against the 96 kDa polypeptide is consistent with it being a major polypeptide antigen in serotype 8 and confirms the earlier observations of Sayed and Kenny (1986) and of Precious *et al.* (1987).

Competition assays with these anti-96 kDa Mabs revealed the presence of at least

4 epitopes and representative Mabs from each of these 4 groups did not show any cross-reactions with any of the other 13 serotypes, suggesting that this 96 kDa antigen is serotype 8-specific. This is the first definite finding of a serotype-specific antigen in any of the 14-serotypes of *U. urealyticum*. This finding disagrees with that of Horowitz *et al.* (1986), who suggested that serotype specificity may be due to quantitative rather than qualitative differences or that non-protein antigens are involved. The anti-96 kDa Mabs thus affords an equivocal differentiation of serotype 8 from the other serotypes and such species-specific probes for all serotypes would allow a better evaluation of the association of individual serotypes in disease processes.

The suppressive effect of an anti-96 kDa Mab on the metabolism of *U. urealyticum*, serotype 8 has been reported previously (Precious *et al.*, 1987). Mabs recognising each of the 4 epitope groups suppressed metabolism of this organism in a similar manner, but multiple epitope binding as determined with combinations of Mabs was not significantly synergistic. The exact role played by this 96 kDa antigen in respect of the growth and metabolism of serotype 8 remains to be elucidated.

Attempts to purify this 96 kDa antigen by immuno-affinity chromatography, co-purified a distinct glycosylated 85 kDa protein, which may be an associated membrane protein in this serotype or could originate from the growth medium. Lin (1982) reported mycoplasmacidal activity of control anti-serum prepared against uninfected growth medium and suggested that medium components were becoming intimately associated with the cell surface and it could be that the 85 kDa protein is one such component. In addition, these medium components may give rise to the extracellular ruthenium-red staining layer observed by Robertson and Smook (1976). Furthermore, if the 85 kDa glycosylated protein were shown to be of medium origin, this could suggest that the 96 kDa antigen may be involved in adherence to epithelial cells through association with a glycosylated component. Again this aspect would merit further investigation.

The 96 kDa antigen would appear to be a surface-expressed, hydrophobic membrane protein with minor hydrophilic characteristics as shown by Bolton and Hunter surface labelling and phase separation with Triton X-114 respectively. It is possible that this minor hydrophilic characteristic is due to the intimate association of glycosylated medium components as described previously, or to a localised domain of hydrophilic components which would allow for easy interaction of the protein with external aqueous environments.

Two Mabs (UU8/39-40), prepared against serotype 8, were shown to recognise two polypeptides of molecular mass 16 and 17 kDa. Unlike the anti-96 kDa Mabs, these had no significant suppressive effect on the metabolism of serotype 8 which suggests that these antigens play a role other than a metabolic one. Difficulties experienced in the purification of these antigens may suggest that *in vivo* these antigens form a close association with other proteins which may make them inaccessible to the Mabs, and so explain the lack of metabolic inhibition. They may also be dependent upon a lipid 'halo' in the membrane such that close association with lipid also restricts binding on the Mab. It is also possible that these antigens play a structural role, as results from phase partitioning suggest that they are exclusively hydrophobic and are most probably integral membrane proteins. Purification of these antigens suggested the close association of other proteins which may suggest these antigens are integral trans-membrane proteins.

Probes which will differentiate the seroclusters of *U. urealyticum*, whilst useful in their own right, would, in addition, obviously assist in investigations to determine whether specific seroclusters predominate in disease processes.

Mab UU8/39 was shown to cross-react with the other 13 serotypes in an interesting manner. The 16 and 17 kDa polypeptides were recognised in all of the serotypes of the large serocluster A, but the 17 kDa polypeptide only was recognised in the serotypes on the smaller serocluster B. On the basis of binding with serotype

13, the latter would appear to be placed in the larger serocluster A. This Mab is the first reported probe which will differentiate the two seroclusters of this organism and to place serotype 13 within one of the seroclusters. Whether the 16 and 17 kDa polypeptides are two separate gene products, one of which has been lost in the smaller serocluster, or whether in the larger serocluster two fairly similar sized polypeptides exist which share a common epitope or whether in the larger serocluster, the 16 kDa polypeptide is derived from the larger is uncertain (see discussion on non-human strains).

Labelling of serotype 8 with ^3H -labelled palmitic acid revealed approximately 26 discrete components, of which 11 were intensively labelled. Similarly, although approximately 25 membrane proteins of *M. capricolum* (Dahl *et al.*, 1983) and 20 proteins of *M. hyorhinis* (Bricker *et al.*, 1988) were labelled with this fatty acid, only 4 proteins of *M. hyopneumoniae* (Wise and Kim, 1987) were so labelled.

It has been reported that fatty acids in the growth medium are utilised primarily for phospholipid synthesis in mycoplasmas and do not serve as substrates for energy production (Dahl, 1988). In *M. capricolum* the major phospholipids are phosphatidylglycerol and cardiolipin and it has been shown that both fatty acid uptake and utilisation in this organism are influenced by the structure of the membrane-associated sterols (Dahl *et al.*, 1981). Since uptake involves both transport and utilisation of the fatty acid, the rate-limiting step for this process in mycoplasmas could be any one in the pathway leading to the end product, phosphatidylglycerol. Current models for fatty acid transport in other prokaryotes suggest that it is protein mediated. In *E. coli*, fatty acid transport is dependent on an outer-membrane protein that binds long chain fatty acid (Black *et al.*, 1987). The fatty acid is then translocated by an unknown mechanism to the cytosolic surface of the inner membrane where it is activated by acyl coenzyme A synthetase. The pathway for phosphatidylglycerol synthesis in *E. coli* proceeds via the dual acylation of glycerol-3-phosphate to yield phosphatidic acid which eventually forms CDP-diglyceride; this is the direct precursor

for glycerolipid synthesis. It is thought that a similar pathway for phospholipid synthesis functions in mycoplasmas (Smith, 1979), but whether this is true for ureaplasmas is unknown.

Of these 26 labelled components, one could be differentiated by immune-precipitation as the 96 kDa antigen. However, several of these proteins were clearly immunogenic as shown by immune-precipitations with homologous polyclonal serum.

The functions of these modified proteins has yet to be determined, although it is clear from this study and from others (Wise and Kim, 1987; Riethman et al. 1987), that they can represent important antigenic structures mediating immune responses as well as possibly playing a structural role in terms of anchorage of membrane proteins (Schmidt *et al.*, 1979) or in determining membrane integrity.

Lipid-modified proteins have been detected in other Mollicutes including *Acholeplasma* and *Spiroplasma* species (Wise and Kim, 1987). In both cases, the proteins were identified as being apparently-surface expressed and this would agree with the surface location of the 96 kDa antigen (as discussed).

The apparent prevalence of lipid-modified proteins among members of the Mollicutes may indeed reflect a general mechanism for the processing and anchorage of critical membrane proteins.

Boyer and Wise (1989) have suggested that these modified proteins are a powerful mechanism for generating antigenic diversity due to the discovery of size-variant lipid-modified antigens in species of *M. hyorhinis* which may be similar to the Streptococcal M proteins. Lipid modification of membrane proteins may also play an important role in dictating intraspecies differences in surface antigenic structure and host interactions and thus may play a role in the pathogenicity of the organism. Whether this is true for ureaplasmas is unknown and the labelling of several other serotypes with fatty acids may be useful to this end.

Active ureaplasma urease was efficiently purified using immuno-affinity chromatography and each preparation, as judged by SDS-PAGE and silver-staining or by urease activity staining, revealed a consistent pattern.

Non-denaturing electrophoresis of purified urease revealed two active bands with apparent molecular weights between 170–200 kDa but it must be recognised that molecular weights cannot be accurately determined in such a system. The use of FPLC, a more definitive technique, suggested a molecular mass for the enzyme of 190 kDa. Previously, various molecular masses have been reported for this enzyme and these include 380 kDa from native gel electrophoresis (Eng *et al.*, 1987; Stemke *et al.*, 1987), 210 kDa (Blanchard *et al.*, 1988), two active bands of around 130 and 180 kDa (Precious *et al.*, 1987) and after treatment with B-mercaptoethanol and SDS, 179 kDa (Eng *et al.*, 1986). Similarly, bacterial ureases from *Brevibacterium ammoniagenes* (Nakano *et al.*, 1984), *Proteus mirabilis* (Jones and Mobley, 1988), *Klebsiella aerogenes* (Todd and Hausinger, 1987) and *Providencia stuartii* (Mulrooney *et al.*, 1988) are reported to all have apparent molecular masses between 200–230 kDa.

Of the two active protein bands observed after SDS-PAGE, that of higher mass was always more active. The band of apparently lower mass probably represents a slightly denatured and hence different conformational state where the sub-units remain associated and enzyme activity retained.

Whilst a partial breakdown of the enzyme into sub-units with associated loss of enzymic activity was seen after freezing of urease preparations at -70°C followed by thawing to 20°C , complete denaturation and total loss of activity was achieved by boiling, even in the absence of denaturing agents. SDS-PAGE of freeze-thawed or of fully denatured enzyme revealed the presence of three sub-units with apparent molecular masses of 72, 14 and 11 kDa. Previous electrophoretic studies on the composition of ureaplasma urease under denaturing conditions (Kenny, 1983; Stemke *et al.*, 1987; Precious *et al.*, 1987; Blanchard *et al.*, 1988) have indicated a polypeptides

of molecular masses variously quoted as being between 64–76 kDa. Whereas this is the first study to demonstrate the presence of the two smaller sub-units, Blanchard *et al.* (1988) have reported the presence of both a 70 kDa and a possible 17 kDa sub-unit.

The three sub-units reported here are similar in mass to the 72, 11 and 9 kDa sub-units reported for the urease from *K. aerogenes* (Todd and Hausinger, 1987), the 73, 10 and 8 kDa sub-units reported for the urease from *P. mirabilis* (Jones and Mobley, 1988) and the 73, 10 and 9 kDa sub-units reported for *P. stuartii* (Mulrooney *et al.*, 1988). The similarity of the urease in both 'native' molecular mass and sub-unit structure between *U. urealyticum* and *P. stuartii* is reflected at the genome level, such that DNA sequences from *U. urealyticum* possess homology with the urease gene from *P. stuartii* as reported by Blanchard and Barile (1988).

On the basis of densitometry, results suggest a hexamer of a_2, b_2, c_2 for the ureaplasma urease, whereas Todd and Hausinger (1987) suggested an a_2, b_4, c_4 complex for *Klebsiella* urease and Mulrooney *et al.* (1988) suggested an $(a_1, b_2, c_2)_2$ for *P. stuartii*. Studies on the genetic organisation of the urease gene(s) from *P. mirabilis* (Jones and Mobley, 1988) and from *P. stuartii* (Mulrooney *et al.*, 1988) suggest the urease peptides are encoded on adjacent DNA sequences and transcribed as a polycistronic mRNA in the order c,b and then a. Whether this is the case for *U. urealyticum* is as yet unknown. It is noteworthy that the urease of *B. ammoniagenes* appears to exist as a trimer of three identical 67 kDa sub-units and that jack bean urease appears to exist as a hexamer of six identical 96.6 kDa sub-units.

Work in this laboratory confirmed that the urease is a nickel containing enzyme with the nickel associated with the 72 kDa sub-unit (Thirkell *et al.*, 1989). This finding is consistent with those for other ureases such as jack bean (Dixon *et al.*, 1975), soybean, *K. aerogenes*, *B. ammoniagenes* and *Bacillus pasteurii* (Todd and Hausinger,

1987) and *Selenomonas ruminantium* (Hausinger, 1986).

The enzyme was shown to have two pH optima of 6.9 and 6.15. These values are not significantly different from pH optima reported by others (Masover *et al.*, 1977; Swanberg *et al.*, 1978) and it is possible that they reflect the two active forms of the enzyme seen after native gel electrophoresis. It was noticeable that the pH of the buffers was lowered after the addition of substrate and this may account for the minor variations in reported values. Although the enzyme is inactivated at pH 8.5 and above, this is reversible since on elution from the affinity column at pH 10.0, followed by dialysis to neutrality, an active preparation is obtained. Dialysis from pH 4.0 to neutrality however, does not reconstitute active enzyme. This irreversible loss of activity has also been observed for jack bean urease and for ureases from *Arthobacter oxydans* and *K. aerogenes* (Todd and Hausinger, 1987).

Kenny (1983) showed that inhibition of urease activity inhibited the growth of *U. urealyticum*. Whereas none of the anti-urease Mabs available inhibited the enzyme or the growth of the organism, it is apparently inactivated by homologous polyclonal antiserum. In addition, Romano *et al.* (1986) reported that ATP production by the cells requires the concomitant activity of both the urease and a membrane bound ATPase. It is unlikely that the cytosolic urease is sensitive to external pH, however, it was noticeable that during batch culture, the external pH rises to and stabilises at around pH 8.1 (Fig 1.1) which is close to the upper limit for enzyme activity. It is also noteworthy that at this pH a plateau is reached and the culture enters a steep lytic decline phase. Thus the enzyme activity may be directly or indirectly related to the extracellular pH, perhaps as a function of the topology of the complex and its relationship to the cell membrane.

After isoelectric focussing of urease stored at 4° C for 48 h, three distinct bands with PI values of 4.60, 4.68 and 4.73 were seen on silver-staining, together with other very minor bands. SDS-PAGE of this preparation showed, with Coomassie stain, the

two active enzyme bands together with the 72 kDa sub-unit (the amount of the two small sub-units present would almost certainly be less than the detection limit of the stain). Fully denatured enzyme preparation, containing the three sub-units only, was shown to have three bands with PI values of 4.68, 4.81 and 4.87. Presumably therefore, the two active enzyme bands have PI values of 4.60 and 4.73, the 72 kDa sub-unit, 4.68 and the two smaller sub-units PI values of 4.81 and 4.87. Whereas Eng *et al.* (1986) have also reported bands with several PI values from their urease preparations, the major of these had PI values between 5.0 and 5.2.

Lead acetate staining of the 'native' ureases from the 14 serotypes suggested that minor variations in molecular mass exist between the serotypes which allowed sub-division into three separate groups. Serocluster A divided into two groups (1:- 2,5,7,8,10,11 & 12; 2:- 4,9 & 13), whilst serocluster B formed a single group. Such variation in the molecular mass of these ureases has been reported previously (Davis *et al.*, 1988).

By two different techniques, competition assays revealed that the urease of serotype 8 possess at least four and possibly five, distinct epitopes, all presumably away from the active site of the enzyme since binding of any of the anti-urease Mabs did not inhibit enzymic activity. In addition, the enzyme was shown to be uninhibited by homologous polyclonal serum at a dilution which had been shown to inhibit the growth of the ureaplasmas (Chapter 3). The unavailability of the active site to the Mabs may suggest that it is protected in a cleft or pocket within the enzyme complex. Against this however, Eng *et al.* (1987) described an antiserum which to varying degrees, inhibited urease activity of several ureaplasma species but which did not inhibit the urease of jack bean or the ureases of several walled bacteria. They argued that the inhibition observed could either be due to the antibody binding directly to the active site, or that the antibody binding induces conformational changes such that the enzyme substrate (urea), albeit of low molecular weight, can no longer react with the enzyme.

Although all 19 anti-urease Mabs bound to the active urease of serotype 8, only 6 of these (recognising three separate epitope groups) consistently recognised the inactive 72 kDa sub-unit. On the other hand, the two smaller sub-units were not recognised by any of the 19 Mabs. Their apparent lack of antigenicity may suggest that they internal within the enzyme complex.

Whilst the 19 anti-urease Mabs also recognised the native ureases of the other 13 serotypes, their 72 kDa sub-units were only recognised by Mabs binding to two of the three above mentioned specific epitopes. The Mabs recognising the third epitope referred to above, did not bind to the 72 kDa inactive sub-units of the ureases of serotypes 3, 6 or 14. This may reflect minor differences in amino acid composition and/or conformation of this sub-unit between the serotypes of *U. urealyticum*.

The hydrophilic nature of the urease enzyme was shown by phase partitioning with Triton X-114 and this is consistent with the cytoplasmic location reported by Vinther (1976) and Masover (1977b). This was confirmed by both Bolton and Hunter labelling and immunogold staining. The staining shown with the latter was similar to that reported by Vinther who used a urea-manganese reagent which in the presence of urease, forms electron dense MnO_2 . Shepard and Howard (1970) suggested that older colonies of ureaplasmas lost urease activity as shown by the urease spot test, and the lack of gold particles within older organisms shown here, would confirm this. The presence of gold particles outwith the cells suggests that this loss of activity is due either to leakage of enzyme from the cells and/or to the release of urease on cell lysis.

Electron microscopic examination of cells grown in a medium containing horse serum revealed large amounts of amorphous material which was not seen when cells grown in a medium containing foetal calf serum were so examined. It is therefore possible that this amorphous material represented inadequately fixed organisms due to the presence of horse serum components adsorbed to the cell surface which may

interfere with the fixation process.

The cells observed ranged in diameter from 0.4 to 0.8 μm although occasionally, much smaller (0.1 μm) and much larger (1.4 μm) cells were seen and such a diameter range is in agreement with the 0.1 – 1.0 μm range published previously (Whitescarver and Furness, 1975). Also as reported previously (Black *et al.*, 1972; Whitescarver and Furness, 1975; Robertson and Smook, 1976), the cells were mostly ovoid or spherical as might be expected for a wall-less microorganism, and they were surrounded by a trilaminar membrane often obscured by a 'fuzzy' layer. The latter may represent the hair-like structures (Black *et al.*, 1972) and/or the ruthenium-red-staining capsule (Robertson and Smook, 1976) which have been described. Robertson and Smook (1976) suggested that the hair-like structures may be a site of adherence for the material stained with ruthenium red. The extramembranous layer has been shown to bind concanavalin A (Robertson and Smook, 1976) which has specificity for glucose and mannose carbohydrate moieties but whether as Lin (1982) has suggested, it originates from the growth medium, remains to be seen. The ability of *U. urealyticum* to bind carbohydrate residues *in vitro* may reflect an *in vivo* affinity for the glycocalyx of epithelial cells of the genital mucosa. It is also possible that only pathogenic strains of ureaplasmas have what may be an adherence virulence factor as variations in this adherence ability have been reported. A bovine strain which when grown under normal *in vitro* conditions, failed to bind ruthenium-red (Robertson and Smook, 1976). Adsorption of carbohydrate-containing material to the exterior of the organism could also play a role in evading the host immune responses if the carbohydrate moiety (presumably from the host) adsorbs to conceal the surface-expressed antigenic determinants.

The mode of reproduction is unclear but many workers (reviewed by Maniloff and Morowitz, 1972) believe that these organisms reproduce by binary fission. Alternatively, Shepard and Masover (1979) suggested that the basic mode of multiplication of *U. urealyticum* was a simple budding process and others

(Whitescarver and Furness, 1975) claimed that two or more buds may appear simultaneously. Here, evidence suggested that these organisms do have the ability to form buds and such a process could explain the short chains of organisms which were observed. It could also be that budding only occurs under certain conditions. Although filamentous reproduction has also been suggested (Black *et al.*, 1972) no evidence for this was seen.

The use of Protein A-gold labelling enabled confirmation of some of the results which have already been discussed. The cytosolic location of the urease was seen and evidence was gained to again suggest that as the cell ages, this important enzyme is lost from the cell. The membrane location of the 96 kDa antigen was confirmed and that the cells were intensely labelled is in agreement with the notion that this is indeed a major antigen of serotype 8. However, this 96 kDa antigen was not accessible to the anti-96 kDa Mabs after fixation with gluteraldehyde and/or polymerisation. It is more likely that this problem is the result of the known cross-linking of components caused by gluteraldehyde fixation but alternatively, it may be that loss of certain epitopes is caused by the polymerisation process. Immunogold labelling also confirmed the membrane location of the 16 and 17 kDa antigens but these were labelled successfully only after permeabilisation of the cells. This suggests that either the epitopes are located on the inside of the membrane or that in the *in vitro* state, these polypeptides are wholly or partly inaccessible because of intimate associations with other membrane components. Were the latter to be the case, this would presumably explain the difficulties experienced when attempts were made to purify these antigens by immuno-affinity chromatography. Treatment with PBS-N would then presumably 'loosen' any such associations to allow recognition of the epitopes by the Mabs and consequently the Protein A-gold.

A similar technique, using anti-rabbit Ig G gold has been used to localise the spiralin and p55 fibrilin protein of spiroplasma cells (Townsend and Plaskitt, 1985).

With the exception of strains of chimpanzee or bovine origin, only a single ureaplasma strain from the cat, dog and marmoset was compared with human *U. urealyticum* serotypes. In general terms, there was a high degree of homogeneity with respect to polypeptide profiles but certain conclusions could be drawn, viz. the bovine, canine and feline strains were distinct from the human, chimpanzee and marmoset strains. Howard *et al.*, 1981 has also claimed 11 bovine strains examined by SDS-PAGE could be distinguished from 8 serotypes of *U. urealyticum*. There were recognisable differences in the polypeptide profiles particularly with respect to polypeptides with apparent molecular weights in the 34-48 kDa range, and there were significant differences in the quantitative amounts of certain polypeptides, most notably those with apparent molecular weights of 166, 60, 28, 25 & 23 kDa.

The canine strain possessed 2 unique polypeptides (21 and 18 kDa) but was otherwise very similar to the feline strain. Although the canine strain had a polypeptide of approximate 96 kDa mass, this polypeptide was not recognised by any of the anti-96 kDa Mabs suggesting that it is unrelated to the 96 kDa antigen of serotype 8. It is possible that this polypeptide plays a similar role to that of the 96 kDa antigen and it would be of interest to discover whether this was a membrane antigen and whether it too was acylated with palmitic acid. Thus the 96 kDa antigen of serotype 8 possesses epitopes which are not expressed in any of the non-human strains examined.

The anti-16/17 kDa Mabs raised against *U. urealyticum* serotype 8 cross-reacted with all of the non-human strains. However, these two polypeptides, as recognised in the serotypes belonging to the larger serocluster of *U. urealyticum* were only recognised as such in all of the chimpanzee strains. Two polypeptides were recognised in the canine strain but these had modified molecular weights of 16.5 and 17.5 kDa. The 17 kDa polypeptide only was recognised in the marmoset strain (similar to the situation with the smaller serocluster of *U. urealyticum* serotypes) whilst the 16 kDa polypeptide only was recognised in the bovine and feline strains. The fact that

one or both of these polypeptides appears to be conserved in all ureaplasmas examined suggests that they play an important role, maybe a structural one as discussed earlier. These findings would also tend to suggest that these are two distinct polypeptides which share a common epitope rather than the 16 kDa polypeptide being a modified product of the 17 kDa polypeptide. Were the latter to be true, it is difficult to explain why even a small amount of the 17 kDa polypeptide was not detected in the bovine and feline strains.

The native urease was detected in all ureaplasmas by gel electrophoresis carried out both in the presence and absence of SDS. Variations in apparent molecular mass may reflect minor variations in amino acid sequence which in turn may alter the structure and/or the conformation of this important enzyme. It was noteworthy that the 72 kDa inactive sub-unit of the urease was recognised in only the chimpanzee, marmoset and feline isolates. It should be reiterated that this sub-unit is not identified on immunoblots of denatured urease from *U. urealyticum* serotypes 3, 6 and 14 with this anti-urease Mab (UU8/17).

The use of the enzyme 'catch test' with anti-urease Mabs recognising five distinct epitopes on the urease appears to confirm that there are differences in the structure and/or conformation of ureases from different species such that certain epitopes are not expressed or are unavailable for binding due to a change in conformational structure.

Many workers feel that the chimpanzee may provide the most appropriate animal model for the study of *U. urealyticum* pathogenicity and with the exception of the 96 kDa antigen (unique to serotype 8), the chimpanzee strains alone gave results similar to those given by the serotypes of *U. urealyticum* of the larger serocluster. This would confirm the view of Mouches *et al.* (1981). With the exception of the results from the catch test, it was also noteworthy that the marmoset strain gave similar results to those given by the serotypes of *U. urealyticum* of the small serocluster.

Although any conclusions would be consolidated by examining a greater number of ureaplasma strains from each of the non-human species, there is sufficient evidence to suggest that the use of immunoblotting with the Mab probes and the catch test enables the species of origin of a ureaplasma strain to be determined.

At the present time, ureaplasmas are studied and diagnosed mainly by those workers in this specialised field. As a consequence, this organism tends to be studied only with respect to its association with certain disease states. Due to the apparent complexities of this organism's pathogenicity, it would be beneficial if these organisms were screened for on a routine day-to-day basis especially in the area of uro-genital infections (and more particularly with respect to non-gonococcal urethritis) and respiratory infections of pre-term low birth weight infants.. Although its role in most of these diseases is no longer disputed, just how important this organism is in these conditions remains to be fully explored. A few years ago, a similar situation existed for chlamydia, but due to the development of enzyme-linked immunoassays and fluorescein-labelled Mabs (Wingerson, 1983), the full extent of this organism's pathogenicity has now been realised. It is now thought that *C. trachomatis* is the single most important cause of pelvic inflammatory disease and its resulting infertility.

Some advances in the production of diagnostic tests for ureaplasmas have taken place recently. Within the last few months a urogenital diagnostic kit has been marketed by D.B.V. Production, France which is based on the Metabolic Inhibition test. It is as yet unproven but relies upon the growth of the strain isolated from a patient (which if not successful can be replaced with freeze-dried antigen) and the patient's own serum to inhibit the growth of the organism. A major drawback to the test is the fact that it remains to be proven that infection with ureaplasma is always associated with a good humoral immune response (other drawbacks to this test have been discussed previously in section 1.1). ELISA systems have been developed (see section 1.1) but these too have drawbacks due to background colour development, presumably due to improper standardisation and cross-reactions among the 14 serotypes. Thus, in

general terms, tests for the diagnosis of ureaplasmas are available only in those laboratories with access to specialised media and reagents and even then, many such tests give arbitrary results and are not quantitative (section 1.1). Any tests applied should be quantitative since only in this way may low-level commensal carriage be distinguished from disease states where larger numbers are thought to be involved (section 1.6).

Due to the success of the fluorescent probes for the diagnosis of chlamydia, an attempt was made to develop such a fluorescent test for ureaplasmas using examples of the three available Mabs linked to FITC as probes.

The probe made with the anti-96 kDa Mab gave good intense specific fluorescence but since the 96 kDa antigen is serotype 8-specific, such a probe would not be of use for preliminary screening of clinical samples. However, such a probe would be useful in secondary screening procedures where serotyping may be important in defining serotypes more specifically implicated with given disease states.

The probe with the anti-16/17 kDa Mab also gave good fluorescence and as either both of these antigens or the 17 kDa antigen alone is present in all 14 serotypes of *U. urealyticum*, the probes with further development could be particularly useful for preliminary screening of clinical samples.

Urease is also present in all serotypes but is both soluble and cytosolic. The FITC-linked probe produced gave much less intense fluorescence with the test material and as a result, difficulties would almost certainly be encountered if this probe were to be tested on clinical material as other components present may mask the low fluorescence seen.

In general, the probes were shown to be both highly specific and sensitive.

Unfortunately, no suitable positive clinical samples were available on which the above probes could be evaluated but there is no reason to suggest that this test

would prove less successful than that for chlamydia. It may also be possible to design a dual probe such that the presence of both ureaplasmas and of chlamydia could be tested for on the same slide using just one sample by tagging the appropriate Mabs with different fluorochromes.

During these studies it was noted that *U. urealyticum* adsorbed specifically to HeLa cells. This adsorption has been reported previously (Manchee and Taylor-Robertson, 1969) where the cells could not be removed by vigorous washing. It is noteworthy that both simian and canine strains of ureaplasmas did not adsorb to these cells. This again indicates the ability of *U. urealyticum* to adsorb to human cells and may or may not play a role in the pathogenicity of disease. Clearly this adsorption to tissue culture cells may provide a suitable model system to study ureaplasma adherence.

The urease catch test, using a plate pre-coated with GAM, proved to be both a simple and reliable test in the laboratory using logarithmic phase cultures of *U. urealyticum*. Unfortunately, the testing of clinical samples was unsuccessful due to the unavailability of either fresh swabs or fresh urine samples. It is obvious from the work here that meaningful clinical trials of the catch test would be dependant upon the immediate use of fresh clinical samples. Attendance at the genito-urinary clinic at the Royal Hospital in Dundee proved pointless as so few appropriate samples were made available during the day.

It would appear that although the catch test is of adequate sensitivity and would not give a positive result in cases of low-level, presumably commensal carriage, urine samples with little or no sediment are unsuitable for testing. However, using the modified dip-stick test, urine samples with little or no sediment could be tested. Both 96-well plates or dip-sticks precoated with GAM and with anti-urease Mab had a shelf-life of at least 4 months but blocking of the plates or dip-sticks with BSA was found to reduce this shelf-life to approximately 2 months.

Slide agglutination tests have the advantage of being quick, easy to read and do

not require reagents other than the Ab-solid matrix conjugate. It appears that the choice of antibody or of antibody combinations is important since only the conjugate with the anti-96 kDa Mab was found to be successful. Even then, the sensitivity was much lower than that achieved, for example, with the catch test in that positive results could not be achieved with logarithmic phase cultures of the organism. The larger numbers of cells required to give a positive result may be a consequence of the small size of the ureaplasma cells. In this case, if ureaplasmas do adhere to cells of the genital mucosa, it is possible that an appropriate slide agglutination test could be developed which would give good positive results with urethral scrapings.

The fact that the 16/17 kDa Mabs did not give positive agglutination may again suggest that these antigens are trans-membrane proteins and unavailable to the Mabs. No agglutination would be expected with a conjugate involving the anti-urease Mabs since the enzyme is cytosolic and would not be available for binding.

Until fresh clinical samples are readily available, the exact success of these various diagnostic tests cannot be thoroughly assessed. There is good reason to believe that at least one of these diagnostic tests, most probably the catch test and the dip-stick test, will be useful and would merit commercial exploitation.

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